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PHENYLALANINE o-HYDROXY-PHENYLACETIC ACID PHENYLPYRUVIC ACID } = PHENYLKETONURIC DIAGNOSIS*

A report of studies made on 77 students in schools for mentally retarded.

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Introduction

Phenylketonuria is a genetically controlled disease classified under the heading of inborn errors of metabolism, and results in severe mental retardation in those affected.

Genetically, the disease is inherited as a recessive trait, equally as frequent in males and females. It appears to be transmitted as a single autosomal gene; therefore, phenylketonurics are probably homozygous having inherited abnormal genes from both heterozygous parents.[6] The incidence of the disease in the United States is estimated to be 4 per 100,000 population at large with Caucasians accounting for almost all reported cases.[3]

Biochemically, the inherited defect has been attributed to the absence or inactivity of an enzyme found in the liver and associated with the metabolism of phenylalanine.

Normally, phenylalanine, an essential amino acid derived from proteins in the diet, is converted to tyrosine. (See Figure 1.) Two protein substances are essential in the oxidative conversion of phenylalanine to tyrosine—a phenylalanine hydroxylase and an accessory enzyme functioning in the cylic regeneration of pteridine-like factor. Studies have shown that only the absence of phenylalanine hydroxylase is concerned in this disease.[5]

As a result of this enzyme failure, the disease is characterized by excretion in the urine of large amounts of phenylalanine and its abnormal metabolites, phenylpyruvic acid and o-hydroxyphenylacetic acid. Testing for the presence of these abnormal metabolites using paper chromatography forms the basis of this study.

It has been suggested that the accumulation of these abnormal metabolites in the tissues is toxic to and interferes with the normal development of the central nervous system.[5] The damage apparently begins shortly after birth as the causative substances may be initially detected in the urine of infants as early as a few days old up to three

* General Diagnostics Student Research Award 1961, and Scientific Products Foundation—Chemistry, 2nd Award 1961. Read before Convention, Seattle, Wash., June 1961.

weeks old. One of the early clinical manifestations of the metabolic defect noted is an abnormal blondism caused by insufficient amounts of melanin.[6] This deficiency is due to the fact that phenylalanine is not converted to tyrosine which is a precursor of melanin. A lack of speech is also evident and behavior patterns may vary widely. Affected persons are classified as either idiot, imbecile or severely retarded with average IQ's of from 18 to 37. Few have an IQ above 70, and the majority of those afflicted are eventually institutionalized.[3]

Early detection of the condition is essential so that dietary treatment may be instituted immediately. By regulating the dietary amount of phenylalanine, the tissue levels of phenylalanine and its abnormal metabolites are held below the toxic level. Encouraging results are reported in which affected infants under eight weeks of age have shown normal development when placed on regulated diets.[5]

The ferric chloride spot test for the detection of the condition is probably the most commonly known. In this reaction, easily conducted in the physician's office or hospital laboratory, the presence of phenylpyruvic acid is checked for by observing the reaction of the urine with ferric chloride. However, due to interfering substances the test may not be conclusive, and results should be confirmed by testing for two other abnormal substances in the urine—the elevated level of phenylalanine and the presence of o-hydroxyphenylacetic acid, which does not appear normally in the urine. Identifying all three of these and especially the two latter ones seems to be essential in confirming the diagnosis of phenylketonuria.

A simple method employing paper chromatography for detecting the presence of these abnormal substances is discussed below. This method was devised by combining and modifying two procedures described by Helen K. Berry in two separate articles.[1,2] This procedure is relatively simple to perform, takes little time, affords clear-cut results and was found to be sufficiently adequate for identifying phenylalanine and o-hydroxyphenylacetic acid in the urine. Phenylpyruvic acid was tested for by using the ferric chloride method.

Method

REAGENTS

Ferric chloride ($FeCl_3 \cdot 10\,H_2O$)
- Ferric chloride ..... 10 Gms.
- Distilled water ..... 100 ml.

Phenylalanine Solvent
- n-Butyl Alcohol ..... 80 ml.
- Glacial Acetic Acid ..... 20 ml.
- Distilled Water ..... 20 ml.

o-Hydroxyphenylacetic Acid Solvent
- Isopropyl Alcohol ..... 80 ml.
- Ammonium Hydroxide, conc ..... 10 ml.
- Distilled water ..... 10 ml.

Ninhydrin
- Ninhydrin ..... 0.2 Gms.
- n-Butyl Alcohol ..... 100.0 ml.

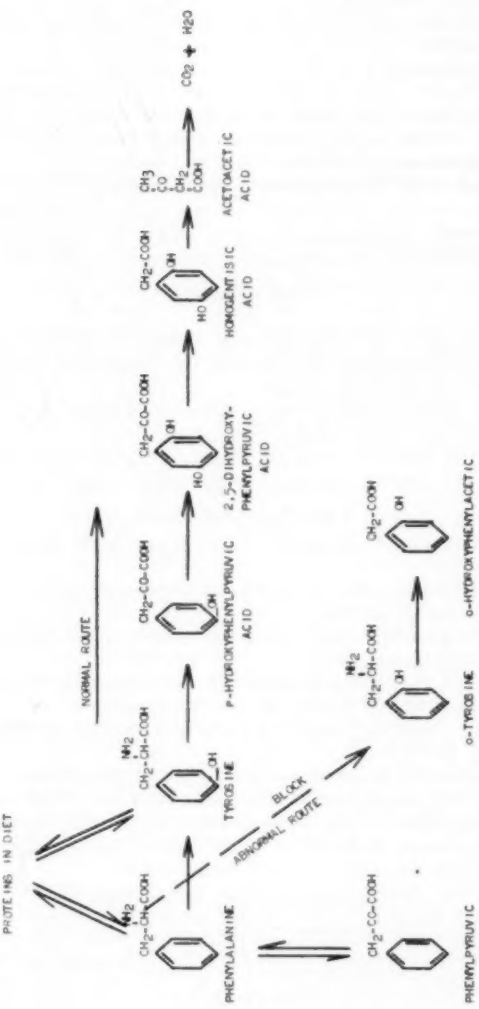

Figure 1—Diagrammatic illustration of normal phenylalanine metabolism and abnormal metabolic products occurring due to enzyme failure in phenylketonuria. (Hawk & Summerson)[4]

Diazotized Sulfanilic Acid

Sulfanilic Acid	4.5 Gms.
Hydrochloric Acid, 12 N	45.0 ml.
Distilled water	200.0 ml.
Sodium Nitrate	4.5%
Sodium Carbonate	10.0%

Dissolve sulfanilic acid in hydrochloric acid and water. Boil. Dilute to 500 ml. Combine equal parts sulfanilic acid and 4.5% sodium nitrate.

Immediately before using, combine equal parts diazotized sulfanilic acid and 10% sodium carbonate.

Phenylalanine and Phenylpyruvic Acid Standard Solutions*

Normal	10 mg/100 ml.
Low elevated	50 mg/100 ml.
High elevated	100 mg/100 ml.

Procedure

Collection:

Collect morning urine specimen in clean containers. 24-hour urine specimens usually required for quantitative tests are difficult to obtain from mentally retarded individuals.

For infants, place heavy filter paper in diapers to be thoroughly soaked, air-dried and forwarded to the laboratory.

Refrigerate the urine if tests are not to be performed immediately as bacterial contamination may alter some of the substances sought.

Phenylpyruvic Acid

Saturate 1-inch square of heavy filter paper with urine specimen and allow to air-dry.

Saturate similar squares of paper with phenylpyruvic acid standard solutions and allow to air-dry.

Apply 3-4 drops 10% ferric chloride solution to both the specimen and the standard paper squares.

A positive reaction is indicated by the immediate formation of a green-colored ring which fades within five minutes.

In a negative reaction there is no color change, the ferric chloride spot remaining yellow.

There are interfering substances which will give color reactions with ferric chloride. Among these are diacetic acid with the formation of a red-colored ring, and certain drug metabolites such as those of aspirin with the formation of a violet or purple color. In certain types of liver disturbances the urine may give a greenish color.

In view of the possibility of these interfering substances, plus the fact that phenylketonurics may occasionally give a false-negative reaction, it would appear advisable to confirm routinely all positive or doubtful reactions for phenylpyruvic acid by the following procedures.

Phenylalanine

In order to follow up the initial findings from the phenylpyruvic acid test, paper chromatography methods for separating the urinary amino acids were used.

* Nutritional Biochemical Corporation, Cleveland 28, Ohio.

Elution Method:

A rectangle of filter paper approximately 1 x ½ inch is saturated with urine and air-dried. One of the smaller sides is cut to form a point.

A wick of heavy filter paper is sandwiched between two glass slides. The pointed tab is placed in contact with the wick by placing the side opposite the pointed end between the slides, overlapping the wick by approximately ⅛ inch.

This apparatus, held together with steel clips is placed in a 50 ml. beaker and distilled water is added up to the bottom of the wick. Water passing up the wick by capillary action allows the water-soluble compounds to be concentrated at the pointed tip of the tab.

Paper Chromatography Method:

Sheets of Whatman #1 filter paper approximately 18 x 11 inches are used for the chromatograms. The concentrated urine is transferred from the tab by applying the pointed tip to the chromatography sheet, producing a circular spot approximately 0.5 to 1 cm. in diameter.

Using this method, ten different urine specimens may be chromatographed simultaneously by spacing the spots at 2 cm. intervals along one 18-inch length of the sheet. A margin of 2 cm. is also allowed between the bottom edge of the paper and the urine spots.

Using the same technique, the standard solutions are applied to the chromatography sheet, providing a means for semiquantitative comparison.

After the spots have dried, the two 11-inch widths of the chromatography sheet are stapled together to form a cylinder without allowing the edges to overlap. This is placed upright in a Pyrex cylinder containing enough phenylalanine solvent to maintain the solvent level approximately ½ inch below the spots. Avoid contact of the chromatography sheet with the sides of the container. The container is made relatively airtight by covering with a piece of plate glass. With this arrangement a solvent-saturated atmosphere is produced within the cylinder. The chromatography equipment necessary may be easily improvised to meet individual circumstances. The sheet is allowed to remain in the solvent 14-16 hours to effect a satisfactory separation.

The paper is then removed and air-dried. Ninhydrin spray (0.2%) is used to develop the color of phenylalanine, which appears as a blue spot approximately 6 inches from the initial application (R_f0.60). The intensity of the colors as compared with the standards is graded. Elevated amounts of phenylalanine in the urine aid in confirming the diagnosis.

o-Hydroxyphenlyacetic Acid

Preparation of the chromatography sheet to detect the presence of o-hydroxyphenylacetic acid is the same as that outlined above for phenylalanine, the only difference being the type solvent system used.

Diazotized sulfanilic acid is used to develop the color in this procedure. o-Hydroxyphenylacetic acid appears as an orange spot approximately 6 inches from the initial point of application (R_f 0.75).

The presence of o-hydroxyphenylacetic acid is specifically diagnostic in phenylketonuria, as normal urines do not show even trace amounts with this method.

Discussion

Using the methods outlined above, urine specimens were tested from 77 individuals in schools for mentally retarded. These individuals ranged from 5 to 26 years of age.

From this study three individuals not previously known to have the condition were positively diagnosed as phenylketonurics. Treatment may be instituted for these individuals at the present time. However, this will only tend to alleviate their condition somewhat, whereas diagnosis and treatment in infancy would have possibly prevented their present state of mental retardation.

The need for wide-spread testing of infants to detect this condition is evident. Although the percentage of occurrence is not overwhelmingly impressive, it IS one of the few mental diseases for which the cause is known and the treatment effective.

The diagnosis should not be allowed to slip by undetected because confirmatory tests were neglected. A positive or doubtful screening test should be succeeded by confirmatory procedures similar to those previously described.

Adaptation of these or suitable procedures by clinical laboratories affords one more medium through which our physicians may combat mental disease.

Summary

A simple, clinical laboratory procedure employing paper chromatography for a positive identification of phenylketonuria is described, based upon the technique of testing for three possible abnormal metabolites found in the urine of these individuals.

Evidence is presented to substantiate further confirmatory tests after a positive or doubtful screening test has been obtained.

Acknowledgment

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USE OF CELLULOSE ACETATE AND PONCEAU S FOR ELECTROPHORETIC SERUM PROTEIN ANALYSIS[1*]

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The basic physicochemical fundamentals of electrophoresis remain poorly understood. Nevertheless this technique is widely used to estimate serum protein fractions. In the last few years a great number of electrophoretic media as well as staining methods have been introduced. Due to the empirical nature of electrophoresis, each modification deserves rigorous examination before unqualified acceptance by the laboratory.

Currently one of the most popular methods of protein electrophoresis entails migration of serum on filter paper and subsequent staining with bromphenol blue. Kohn[1] has recently proposed cellulose acetate strips as a suitable supporting medium for serum electrophoresis. This worker has also suggested Ponceau S[2] as a protein stain. As yet relatively little information is available concerning the use of either cellulose acetate or Ponceau S for serum protein measurements. Therefore, the technique of Kohn[1,2] with some modification has been evaluated in our laboratory and compared to electrophoretic fractionations performed on filter paper and stained with bromphenol blue.[3]

MATERIAL AND METHODS

Electrophoresis on filter paper. Eight paper strips (S & S 2043A) were placed in a Durrum-type cell attached to a Duo-stat power supply (commercially available from Spinco Division of Beckman Instruments, Belmont, California). Veronal buffer of pH 8.6, ionic strength 0.075 was used to fill the cell compartments and saturate the strips. At least 15 minutes was allowed for equilibration of strips with the water-vapor atmosphere of the chamber. Each strip was streaked with 6μl. of serum and a constant current of 3 mA. was applied to the cell for 16 hrs. Strips were then removed and dried for exactly 30 minutes at 120° C in a constant temperature oven. Afterwards strips were placed in methanol for several minutes and transferred to a solution containing 1.0 gm. bromphenol blue (Spinco B-4 dye) dissolved in 1 l. methanol. After 30 minutes the strips were rinsed for 6 minutes each in three separate 5% aqueous solutions of glacial acetic acid. Strips were then dried for 15 minutes at 120° C in an oven and subjected to ammonia vapor to develop color. The length of strip occupied by each protein fraction was measured, cut and placed in 6.0 ml. of 0.01 N NaOH for 1 hr. Due to high color intensity segments containing serum albumin fraction were routinely treated with 12.0 ml. of dilute alkali. Protein-free areas of each strip were eluted to serve as paper blanks. All readings were made against a reagent blank in a Coleman Universal Spectrophotometer at 540 mμ using Coleman cuvettes (19 × 105 mm.). The optical densities of dye-containing strips were corrected by subtracting the paper blank

*This investigation was supported in part by the United States Public Health Service (Project A-4609).

for the appropriate size of the segment. The percentage dye distribution was calculated from the corrected optical densities obtained for each protein fraction.

Electrophoresis on cellulose acetate. Sheets of cellulose acetate (Oxoid No. 11-53G) were impregnated with a solution of 8.8 gm. of barbitone acetate buffer (Oxoid No. BR 11-G) dissolved in 1 l. distilled water. This was accomplished by floating cellulose acetate strips on the buffer. After saturation strips were completely immersed in buffer for a few minutes, then blotted lightly and placed in a Shandon electrophoretic cell (commercially available from Consolidated Laboratories, Inc., Chicago Heights, Illinois). This cell accommodates 4 strips which lie horizontally and connect two compartments containing barbitone acetate buffer. A 10 μl. aliquot of serum was applied in the middle of each strip as a streak. Care was taken not to extend serum application to the sides of the strip. A constant current of 0.4 mA/cm. width of cellulose acetate was applied to the cell for 3 hrs. Strips were then removed, air dried and floated right side up on the surface of a 0.2% solution of Ponceau S (Oxoid No. 14-129) for 5 to 10 minutes. After penetration from below was complete, strips where immersed in the stain for another 5 minutes. Strips were then rinsed in 5% aqueous solution of glacial acetic acid until a clear background was obtained. A final rinse in distilled water removed all traces of dye not bound to protein (Fig. 1). After air drying, the stained areas occupied by each protein fraction were measured, cut

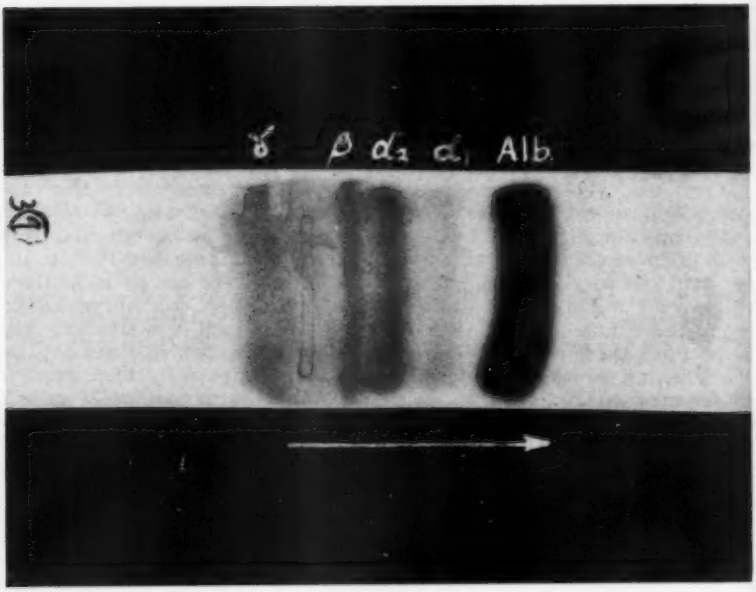

Fig. 1—An electrophoretic pattern obtained from 10 μl. of serum separated on cellulose acetate and stained with Ponceau S.

and placed in 6.0 ml. of 0.1 N NaOH. Serum albumin fractions were routinely treated with 12 ml. of 0.1 N NaOH. Tubes were inverted several times during a 15 minute elution period. Optical densities of eluates were determined in a Coleman Universal Spectrophotometer at 560 mμ. The percentage dye distribution was calculated in a manner similar to that employed for strips stained with bromphenol blue.

RESULTS AND DISCUSSION

Absorption curve of Alkaline Ponceau S. In order to determine the optimal wavelength for measuring eluted Ponceau S the absorption curve of an alkaline solution was established. This was achieved by staining a cellulose acetate strip on which 4 μl. of serum had been applied. The protein-bound stain was eluted with 12 ml. of 0.1 N NaOH. Ponceau S in alkaline solution is purple. The optical densities of this solution were determined between 350 mμ and 800 mμ at 10 mμ intervals using a Coleman Universal Spectrophotometer against a reagent blank (Fig. 2). Maximum absorbence occurred between 550 and 570 mμ. Meulemans[5] has suggested that alkaline solutions of Ponceau S be quantitated at 550 mμ. However, slightly higher optical densities may be obtained by making reading at 560 mμ.

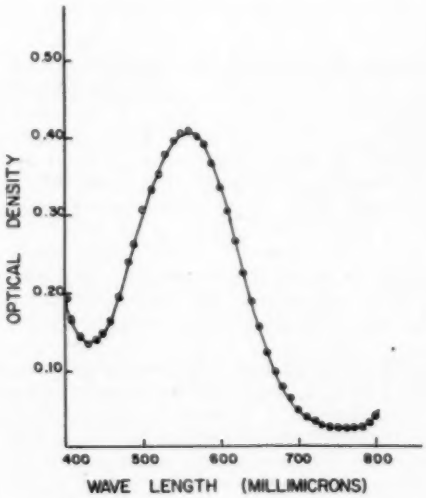

Fig. 2—Absorption curve of an alkaline solution of Ponceau S.

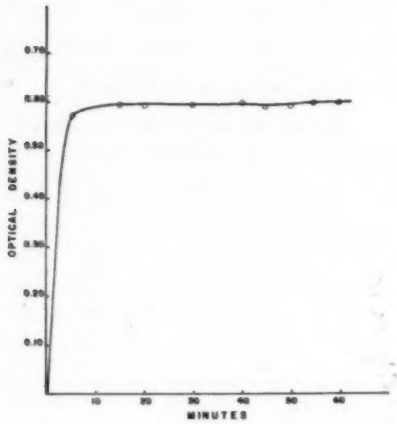

Fig. 3—The effect of time on elution of Ponceau S from serum applied to cellulose acetate strips.

Elution of Ponceau S from cellulose acetate. A 4 μl. aliquot of pooled sera was applied to cellulose acetate paper, stained with Ponceau S and placed in 12 ml. of 0.1 N NaOH. Elution of dye was studied at 7 time intervals ranging from 5 to 60 minutes. Each tube was inverted several times during elution. Optical densities were then measured at 560 mμ in the spectrophotometer. Elution of dye from the strips was observed to be extremely rapid (Fig. 3). A near maximum optical density was obtained after treating stained strips with alkali for only 5 minutes. Little change in optical densities occurred between 15 and 60 minutes. On the other hand, Henry et al.[3] found that bromphenol blue extracted in 0.1 N NaOH faded appreciably in 1 hr. When 0.01 N NaOH was used for bromphenol blue extraction fading was no greater than 2% within 1 hr.

Dye-binding with increasing protein concentrations. Aliquots of pooled sera ranging from 2 to 8 μl. were applied to cellulose acetate and stained with Ponceau S. A duplicate series of serum aliquots was placed on filter paper and treated with bromphenol blue. The amount of stain bound by serum was then measured colorimetrically. The amounts of Ponceau S recovered followed Beer's Law at the serum concentrations studied (Fig. 4). On the other hand, recovery of bromphenol blue was non-linear with increasing serum concentrations. Other workers[3,4] have also observed significant deviations from Beer's Law when bromphenol solutions were quantitated colorimetrically.

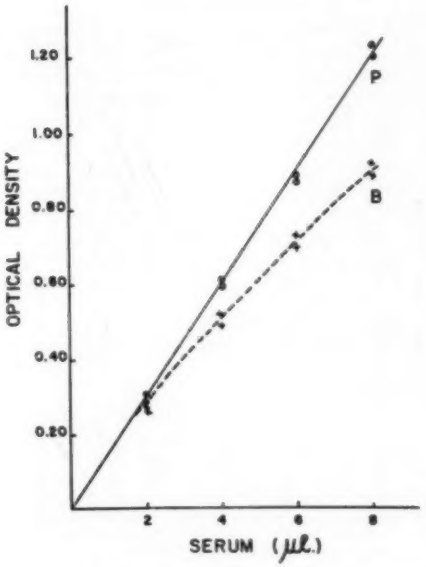

Fig. 4—The uptake of Ponceau S (P) and bromphenol blue (B) by increasing amounts of serum.

Reproducibility of the Ponceau S Technique. Serum proteins were separated electrophoretically on cellulose acetate from a randomly selected serum sample on 4 separate occasions. After staining with Ponceau S the percent distribution of dye in each protein fraction was compared to the mean of 4 determinations (Fig. 5). The range of values appears within acceptable limits for each electrophoretic component. For example, the dye-uptake by serum albumin ranged from 64 to 67% with a mean value of 66%. Ranges obtained for the other protein fractions were equally satisfactory.

Comparison of the two electrophoretic methods. Sera collected from 8 randomly selected individuals were subjected to electrophoresis on both cellulose acetate and filter paper. Proteins separated on cellulose acetate were stained with Ponceau S while filter paper strips were treated with bromphenol blue. The two staining techniques yielded slightly different results for some protein fractions (Table 1). For example, the beta globulins tended to bind a greater percentage of bromphenol blue than Ponceau S. On the other hand, a greater proportion of Ponceau S was contained in gamma globulins. Muelemans[5] has recently compared the dye binding of Ponceau S and azocarmine B to electrophoretically separated proteins. Ponceau S was found to have the same dye-binding capacity for albumin and globulins in contrast to the azocarmine which requires the use of a correction factor in evaluating protein fractions.

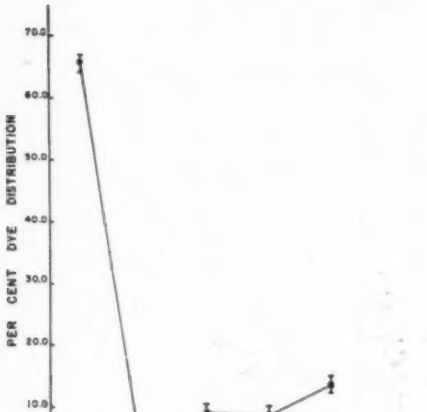

Fig. 5—Replicate measurements of Ponteau S dye distribution in serum protein fractions.

TABLE 1

Serum Number	ALBUMIN		ALPHA–1–GLOBULIN		ALPHA–2–GLOBULIN		BETA–GLOBULIN		GAMMA–GLOBULIN	
	P[1] (%)	B[2] (%)	P (%)	B (%)	P (%)	B (%)	P (%)	B (%)	P (%)	B (%)
30.....	68.1[3]	65.5	3.1	4.0	8.2	9.0	7.3	10.5	12.1	10.8
34.....	63.8	59.4	2.8	3.0	9.3	9.2	7.9	12.9	16.7	15.4
63.....	70.0	75.3	2.7	2.4	7.5	5.4	7.3	8.0	12.8	9.0
24.....	70.2	67.0	3.3	3.2	8.6	9.6	7.4	10.4	11.6	10.0
17.....	61.1	71.0	3.7	2.6	9.8	8.6	9.6	8.7	15.6	9.3
23.....	31.4	31.8	6.6	5.5	12.1	10.9	10.5	13.7	39.3	37.1
39.....	67.1	64.5	2.1	2.7	8.9	6.8	9.3	12.5	13.4	13.3
40.....	50.4	53.5	3.6	3.5	13.7	12.9	10.2	11.3	20.2	18.8

[1] Ponceau S Dye.
[2] Bromphenol Blue Dye.
[3] Each figure represents the mean of two separate determinations.

Table 1—Distribution of Ponceau S and bromphenol blue on serum protein electropherograms

CONCLUSIONS

The use of Ponceau S to stain serum separated electrophoretically on cellulose acetate has been evaluated and found to be an efficient and reproducible method for estimating proteins. The percentage of dye in protein fractions has been determined by elution in 0.1 N NaOH and subsequent colorimetric quantitation in a Coleman Universal Spectrophotometer at 560 mμ. The relationship of increased protein concentrations to dye-binding capacity was linear when aliquots of a pooled serum ranging from 2 to 8 μl were employed. Little changes in optical densities of eluates occurred during a 60 minute interval. Comparisons with a bromphenol blue staining technique suggest that the amount of dye-uptake by protein fractions is dependent upon both the electrophoretic medium and choice of stain.

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ABSTRACTS

LABORATORY AIDS IN THE DIAGNOSIS AND TREATMENT OF URINARY TRACT INFECTIONS

Pyelonephritis and related infections of the urinary tract are among the most frequently undiagnosed and most difficult to manage of all infectious diseases. It becomes readily apparent that the typical symptoms and signs alone are not to be depended upon in the diagnosis of pyelonephritis.

Laboratory methods that may be of some value in the diagnosis and treatment of pyelonephritis are:

1. **Urinalysis.** The first morning voided specimen is best as it will have been in the bladder overnight and any bacteria present will have multiplied. The Gram stain or methylene blue stain of the urinary sediment for bacteria are extremely valuable diagnostic procedures. These stains are usually positive when 100,000 or more organisms per ml. are present.

2. **Glitter-cell Phenomenon.** These cells are polymorphonuclear leukocytes which stain a pale blue with a gentian violet-safranin stain and contain granules in the cytoplasm which exhibit Brownian movement. Glitter-cells are frequently present in pyelonephritis, but are not pathognomonic.

3. **Griess's Test.** The majority of organisms producing urinary tract infections will reduce to nitrites the nitrates normally present in urine. The Griess reagent contains sulfanilic acid, acetic acid and alpha-naphthylamine. When added to a specimen of urine containing nitrite, it rapidly turns red because of the formation of azo-alpha-aminonaphthaline parabenzine-sulfonic acid. Uninfected urine contains no nitrite and does not change in color. The chief drawback to Griess's test is its insensitivity—a significant bacilluria can be present and the test may be negative.

4. **Urine Culture.** The use of a quantitative urine culture provides a method by which true bacilluria can be differentiated from contamination. Significant bacilluria is present when 100,000 or more bacteria are found in one ml. of urine. When less than 1,000 per ml. are present they are contaminants, while organisms present in the range of 1,000 to 10, per ml. are of variable significance. Bacteriostatic agents, water diuresis, lack of multiplication in the bladder and obstruction of the ureters all affect the results.

5. **Antibody-hemagglutination Response.** This method has been used with patients having urinary tract infections, the results providing some success. Red corpuscles from the patient are mixed with a dilution of a specially prepared bacterial antigen which is adsorbed on the corpuscles. Then the hemagglutination test is performed, using the patient's serum at various dilutions from which the antibody titer is obtained.

It is hoped that the utilization of these tests will facilitate the detection of asymptomatic pyelonephritis and assist in the therapy of this important renal disease. Straffon, R. A., Department of Urology, Cleveland Clinic, Cleveland, Ohio. *Cleveland Clinic Quarterly,* 28, 1, 1961, pp. 30-37.

THE CONCENTRATION OF TUMOUR CELLS IN BLOOD

A simple, reliable and quantitative technic is described. First, the polymorphonuclear neutrophils were removed by allowing them to ingest corbonyl iron particles, then removing this iron with a strong magnet. During the removal of the iron, the red corpuscles were lysed by saponin, following which the fluid was centrifuged to deposit all the lymphocytes and any tumor cells which might have been present. These cells were then fixed in the free state, and the fixative containing the cells filtered through a membrane filter which, with the cells embedded in it, was then handled like a frozen section. It was stained in hematoxylin and eosin prior to mounting on a slide. Using this technic, all the tumor cells from 5 ml. of blood were concentrated on a single filter of 47 mm. diameter, the whole of which was examined microscopically. Luckcock, E. D., Institute of Diseases of the Chest, Brompton Hospital, London, S. W. 3, England. *The Journal of Medical Laboratory Technology,* 18, 1, 1961, pp. 32-35.

THE DETERMINATION OF BLOOD GLUCOSE AND UREA NITROGEN ON A SINGLE MICRO-SAMPLE†

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Abstract

A micro-method for the analysis of blood glucose and urea nitrogen utilizing one 0.2 ml sample of whole blood, plasma, or serum, is presented. After the initial micro-measurement, all further steps utilize macro-measurements and routinely available laboratory equipment.

The proposed method has a coefficient of variation of 2.0% and 2.3% for blood glucose and urea nitrogen respectively. For 27 healthy and fasting adults, the blood glucose plus or minus 1 standard deviation was 83.2 ± 10.8 mgm% and for blood urea nitrogen was 14.2 ± 3.4 mgm%.

There is a need for micro-chemical methods which employ only one micro-measurement and will utilize commonly existing laboratory equipment and facilities. The basic desire is to make an initial 0.1 or 0.2ml volumetric measurement of a sample of blood, serum, or plasma. The method should yield sufficient filtrate or diluent so that future steps can utilize macro-measurements.

The following material is an evaluation for a procedure to determine both blood glucose and urea nitrogen with one micro-sample of blood, serum, or plasma. The glucose oxidase[2, 6] method and the diacetyl monoxime[1, 4, 5] method are used for the analyses. Minor variations are necessary to adapt these procedures to a micro-chemical approach.

Sahli-type pipettes* by which 0.1 and 0.2ml quantities can be readily measured with an accuracy of $\pm 0.5\%$ fulfill the primary requirement of the proposed method. These pipettes are durable, easy to handle, and quite inexpensive.

Experimental Design

The analyses are done on plasma obtained from venous blood. The normal values are obtained from healthy young adults in a fasting condition. Chemical standards, control serums, and two to three unknowns are run daily. A commercial synthetic serum** is used as a control and for the determination of day to day variation in the method.

The glucose enzyme analysis is not comparable to Cu^{++} reduction methods.[6] Therefore the blood glucose values are presented without comparison. Several authors[2, 6, 7] have suggested analyzing blood for glucose on aqueous dilutions of plasma or serum. For this reason, a comparison is made between the plasma protein-free filtrate and aqueous dilution.

The monoxime method is compared with the Conway micro-diffusion procedure.[3] The diffusion technique is free of the objections leveled against steam distillation, direct and indirect Nesselerization, and direct colormetric methods. Therefore, the Conway method can be used as a check on other urea analytical techniques.

† The views and opinions expressed in this paper are those of the author and are not to be construed as a statement of policy of the United States Air Force.

* Alpha-pette, Richard Allan Co., Inc., 1335 Dodge St., Evanston, Ill.
** Labtrol, Dade Chemical Corp., Miami, Florida.

Received for publication April 1961.

Reagents

a. Reagents for filtrate:
   1. Prepare 1000 ml. of 0.15 M (approx. 5% w/v) $ZnSO_4 \cdot 7H_2O$.
      Weigh 50 gms. of reagent grade $ZnSO_4 \cdot 7H_2O$ and dissolve to 1000 ml. with distilled water.
   2. Prepare 1000 ml. of 0.15 M (approx. 5% w/v) $Ba(OH)_2 \cdot 8H_2O$.
      Weigh 50 gms. of reagent grade $Ba(OH)_2 \cdot 8H_2O$ and dissolve to 1000 ml. with distilled water. Store in refrigerator.
   3. These two solutions must be equivalent to one another.
      Titrate slowly 25 ml. of the $ZnSO_4$ solution with the $Ba(OH)_2$ to the phenolphthalein endpoint. Correct the appropriate solution with dilution until equivalence is obtained.

b. Reagents for glucose oxidase:
   1. Glucose oxidase reagent (Glucostat)†
      The reagents are supplied dry in 2 vials. The small vial (labeled Chromogen) is dissolved with 1.0 ml. of methanol and added to 50 ml. of distilled water. The contents of the large vial (labeled Glucostat) are dissolved with small portions of water and added to the 50 ml. Chromogen solution until the volume is 80 ml. The dilute reagent is stable for 24 hours at room temperature. The Glucostat is available in 4x size, making 320 ml. of dilute reagent. Aliquots of dilute reagent can be frozen and remain stable for 14 days. Auto-oxidation of the Chromogen (o-dianisidine) gives the solution a gradually increasing brown color.
   2. 4M Hydrochloric acid.
      Concentration is approximate. Mix 28 ml. of concentrated HCl with 72 ml. of distilled water.
   3. Stock glucose standard—1.00 gm. per 1000 ml.
      1.00 gm. of reagent grade glucose is dissolved in and made to 1000 ml. with 0.1% w/v solution of benzoic acid.
   4. Working glucose standard—2.0 mgm. per 100 ml.
      2.0 ml. of stock standard are diluted to 100 ml. with distilled water. Prepare working standard fresh every other day.

c. Reagents for urea nitrogen analysis:
   1. Diacetyl monoxime—2.5% w/v.
      Dissolve 2.5 gms. of 2-3 butanedione - 2-oxime to 100 ml. with 5% v/v acetic acid solution. Store in brown bottle.
   2. Stock Arsenic-Hydrochloric acid solution.
      Dissolve 50.0 gms. of reagent grade arsenic acid to 500 ml. with concentrated HCl. Work with solution under a hood.
   3. Working Arsenic-Hydrochloric acid solution.
      Dilute 67 ml. of stock solution to 250 ml. with distilled water.
   4. Stock urea nitrogen standard-20 mgm per 100 ml.
      Weigh accurately by difference 225 mgm. of urea and dissolve to 500 ml. with distilled water.
   5. Working urea nitrogen standard—1.0 mgm. per 100 ml.
      Dilute 5.0 ml. of stock urea standard to 100 ml. with distilled water.

Equivalent concentration of urea nitrogen $= \frac{\text{weight urea}}{10} \times \frac{28}{68}$ mgm.%

† Glucostat, Worthington Biochemical Corp., Freehold, N. J.

Procedure

a. Preparation of filtrate:

1. Collect blood by venipuncture and place 5 ml. in tubes containing 0.010 gms. of lithium oxalate. Centrifuge and separate off plasma.
2. Rinse a 0.2 ml. sample of plasma or control serum into 9.0 ml. of distilled water in a 13 × 100 mm. test tube.
3. Add 0.5 ml. of $Ba(OH)_2$ solution, mix, then add 0.5 ml. of $ZnSO_4$ solution, and stopper. Shake violently for 10 seconds and let stand until a flocculent precipitate appears. Filter through 7 cm. in diameter Whatman No. 40 filter paper.
4. For direct glucose analysis, rinse 0.2 ml. of plasma into 10.0 ml. of distilled water.

b. Analysis of glucose:

1. Into cuvettes (19 mm. in diameter Coleman)‡ add solutions according to the following scheme.

Solution	Unknown	Standard	Control	Blank
Filtrate	1.0 ml.	—	1.0 ml.	—
Water	—	—	—	1.0 ml.
Standard	—	1.0 ml.	—	—

2. To each tube add 5.0 ml. of glucose enzyme reagent.
3. Incubate tubes at 37± 1.0° C. for 30 ± 0.5 minutes in waterbath.
4. Immediately add 1 drop of 4M HCl to each tube and shake gently.
5. Read Optical Density (O.D.) of each tube against the blank at 400 mu.
6. Calculate unknown concentrations by following formula.

$$\text{concentration} = \frac{\text{O.D.}_{\text{unknown}}}{\text{O.D.}_{\text{standard}}} \times 102 \text{ mgm.\%}$$

a. Analysis of blood urea nitrogen:

1. Into 15 × 125 Pyrex test tubes add solutions according to the following scheme.

Solution	Unknown	Standard	Control	Blank
Filtrate	5.0 ml.	—	5.0 ml.	—
Water	1.0 ml.	4.0 ml.	1.0 ml.	6.0 ml.
Standard	—	2.0 ml.	—	—

2. Add 3.0 ml. of working arsenic-hydrochloric acid solution to each tube.
3. Add 1.0 ml. of diacetyl monoxime solution to each tube and mix.
4. In subdued light, incubate all tubes in water bath at 100° C. for 15 ± 0.1 minutes.
5. Remove from boiling water bath and immediately immerse in cold water bath at a maximum of 15° C.
6. Cool for 5 minutes and then read optical density of each tube contents in a cuvette (19 mm. in diameter Coleman) against the blank at 475 mm. Color stable for 20 minutes after cooling.
7. Determine concentration of unknown from calibration curve.

$$\text{BUN} = \text{result} \times 1.02$$

Calibration Curves

a. Glucose:

1. Dilute stock standard, 10.0 ml. to 100 ml., with distilled water.

‡ Coleman Spectrophotometer Jr., Coleman Instruments, Inc., 42 Madison Ave., Maywood, Ill.

2. Use the following scheme to obtain several concentrations of glucose.

Volume of 10 mgm.% Solution	Volume of H_2O	Equivalent Conc.
6.0 ml.	4.0 ml.	300 mgm%
5.0 ml.	5.0 ml.	250 mgm%
4.0 ml.	6.0 ml.	200 mgm%
3.0 ml.	7.0 ml.	150 mgm%
2.0 ml.	8.0 ml.	100 mgm%
1.0 ml.	9.0 ml.	50 mgm%
0.5 ml.	9.5 ml.	25 mgm%

3. Use 1.0 ml of above dilutions and proceed as under glucose analysis. Plot optical density vs. concentration.
4. The calibration curve is based on a 1/50 dilution. Since the unknowns and control are a 1/51 dilution, the values obtained from the calibration curve are multiplied by 51/50 or 1.02. From figure 1 the enzymatic determination of glucose obeys Beer's Law.

b. Blood urea nitrogen:

1. Add the following volumes of working standard and water to 15 × 125 mm. Pyrex test tubes.

Volume of Working Standard	Volume of H_2O	Equivalent Conc.
0.5 ml.	5.5 ml.	5 mgm%
1.0 ml.	5.0 ml.	10 mgm%
2.0 ml.	4.0 ml.	20 mgm%
3.0 ml.	3.0 ml.	30 mgm%
4.0 ml.	2.0 ml.	40 mgm%
5.0 ml.	1.0 ml.	50 mgm%
6.0 ml.	0.0 ml.	60 mgm%

The precise equivalent concentration depends upon concentration of stock standard. *Here assumed to be 20 mgm%.*

2. Proceed as under the analysis of urea with the addition of the arsenic-hydrochloric acid solution.
3. Plot optical density (O.D.) vs. concentration. Notice the curve is not linear.
4. As with the glucose calibration, unknown values are multiplied by 1.02. As seen in figure 1, the direct colorimetric analysis of urea does not follow Beer's Law. The curve is non-linear for values less than 10 mgm%. However, the points on the curve are quite reproducible.

Reactions

Both methods require timed incubation periods. The reaction rate curves are plotted in figure 2.

For glucose, the reaction at thirty minutes is virtually complete and small errors in timing are not critical. A shorter incubation time gives reproducible results but requires considerable precision in timing. In emergency determinations, a 10 minute incubation period may be useful. The temperature of the water bath should be maintained within ±1.0° C. This factor is particularly important if the laboratory uses a mean

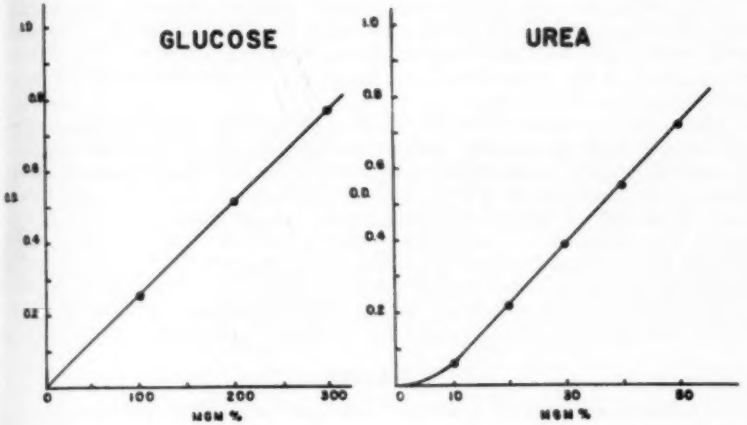

Figure 1—The mean calibration curves for glucose and urea nitrogen are based on chemical standards. Notice Beer's Law is not obeyed for the direct colorimetric analysis of urea nitrogen.

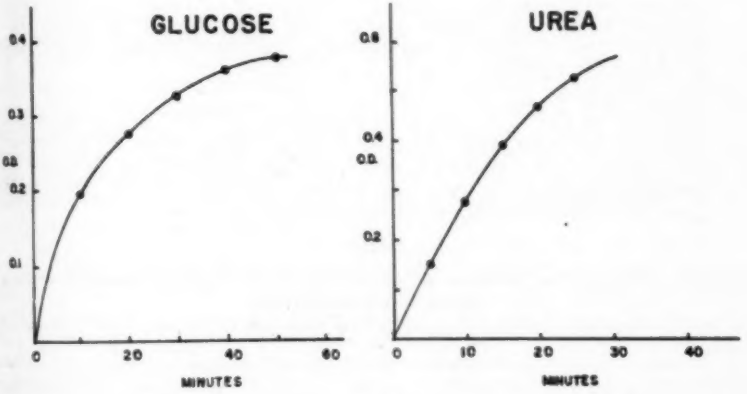

Figure 2—The reaction rate curve for glucose is based on an equivalent 125mgm% solution of glucose. For urea nitrogen, the curve is based on mgm% solution.

calibration curve for obtaining results. Greater variations in temperature cause a rather large increase in the variance of the procedure. This effect can be minimized by calculating results from optical density ratios using a daily chemical standard.

Other methods use smaller volumes of glucose enzyme reagent than proposed here. By using 2.0 or 3.0 ml. of reagent, there is a problem with non-linear and non-reproducible calibration curves. With 5.0 ml. of reagent, these inconsistencies disappear. Apparently Cawley et al. experienced similar findings.

The urea-monoxime complex shows a continual rapid change in optical density with increasing time of incubation. The fifteen minute point is selected to give a calibration curve having a practical range of values. The rapid changes in optical density with time requires considerable care in timing of the incubation in order to minimize the overall variance. The reaction is moderately photosensitive[1] and during heating should be shielded from direct light. Once the reaction is cooled below 30-40° C., the absorbance remains stable for at least 20 minutes in either incandescent or fluorescent light. To some laboratory personnel the reaction has an objectionable odor and is best worked with under a hood.

Results

a. Variation and Error:

1. Glucose.

The day to day variation in the serum control and the chemical standard are summarized in Table 1. The results are based on the analysis of the control synthetic serum and working glucose standard for 10 consecutive days. The analytical values are obtained from a mean calibration curve with a $k = 372 \pm 10$ at 2 standard deviations. (k = conc. glucose/optical density).

For glucose, the day to day variation was such that 95% of all determinations on the control fell within ± 6.2 mgm% of the mean concentration of the control. The standard error of the mean is 1 mgm%. The analysis of the chemical standard shows a coefficient of variation of 2.0%.

2. Urea nitrogen.

The day to day variation is summarized in Table 1. As with glucose, the analyses were done for 10 consecutive days. For comparison, the Conway micro-diffusion method was used to analyze the synthetic control serum. The values obtained for the monoxime method are from a mean calibration curve.

TABLE 1

Summary of Day to Day Variation in Control Serums and Chemical Standards Analyzed run for 10 consecutive days

Glucose	Mean	s.d.
Control Serum	94.1 mgm %	3.2 mgm %
Standard-100 mgm %	100.4 mgm %	2.1 mgm %
Urea Nitrogen		
Control Serum (Conway method)	15.6 mgm %	0.7 mgm %
Control Serum (Monoxime method)	15.7 mgm %	0.5 mgm %
Standard-21.2 mgm % (Monoxime method)	21.0 mgm %	0.5 mgm %

For the urea nitrogen, the day to day variation in the monoxine method was such that 95% of all determinations on the control fell within ± 1.0 mgm% of the mean concentration of the control. The standard error of the mean is 0.15 mgm%. The analysis of the chemical standard shows a coefficient of variation of 2.3%.

The comparison between the Conway method and the monoxime method shows by inspection that the values are from the same sample population.

b. Normal Values:

1. Glucose.

The average concentration of plasma glucose in fasting young adults and the comparison between the protein-free filtrate and aqueous dilution are summarized in Table 2. The difference between these two results is significant having a $p = 0.002$.

2. Urea Nitrogen.

The average concentration of plasma urea nitrogen in fasting young adults as analyzed by the monoxime and micro-diffusion methods is summarized in Table 2. The difference between these groups is not significant as can be seen by inspection.

TABLE 2

Summary of Range of Normal Values in 27 Healthy Adults

Glucose	Mean	s.d.
Plasma filtrate	83.2 mgm %	10.8 mgm %
Plasma aqueous	74.0 mgm %	9.8 mgm %
Urea Nitrogen		
Monoxime method	14.2 mgm %	3.4 mgm %
Conway method	14.0 mgm %	2.9 mgm %

Discussion

The proposed method offers a micro-chemical technique which is reasonably accurate, rapidly performed, and produces results that are in a commonly accepted range. While this evaluation used venous plasma, it is perfectly feasible to obtain capillary blood from a finger prick. The 0.2 ml. pipettes are easily filled with whole blood and rinsed directly into a 9.0 ml. aliquot of water previous to making a protein-free filtrate. During the study, 16 of the 27 individuals used as normals were simultaneously analyzed by both venous plasma and capillary blood samples. While not reported here the data from capillary blood filtrates and plasma filtrates were in close agreement.

The use of aqueous dilutions of plasma or serum for the determination of glucose gives data that is significantly lower than those obtained from protein-free filtrates. Such a finding is in agreement with Saifer and Gerstenfeld[6] but not with Cawley et al.[2] An interesting factor is the degree of dilution. As increasing dilutions of plasma are used, the difference from protein-free filtrate reaches a plateau of minus 8-9 mgm%. Cawley et al[2] found a similar phenomenon but showed that dilutions in the area of 1/50 result in values significantly comparable to a protein-free filtrate.

The effect of dilution is to reduce the concentration of inhibitors.

Others[2,6] have studied the inhibitors of the glucose oxidase enzyme. The important ones are uric acid and hemoglobin pigments. While dilution tends to reduce their effect, a protein-free filtrate can only remove them completely. Certainly the difference is small and for practical work, particularly in emergencies, the speed offered by doing analyses on an aqueous plasma or serum dilution is worth considering.

In studying the glucose oxidase system, I observed two other interesting features. It is common knowledge, that on standing, whole blood and plasma sugar levels fall inconsistently but significantly. This effect is minimized in routine procedures by making a protein-free filtrate as soon as possible after obtaining the sample. In doing direct dilution analyses this change must be considered. After separation and dilution, the plasma from the 27 individuals in the study was measured for glucose content at 1, 2, and 4 hours. Within this time period, no difference was observed.

I also did a small pilot study to get an indirect approach to the specificity of the enzyme. Different neutral protein-free filtrates of a pooled serum were made. These filtrates were: $CuSO_4$-Na_2WO_4, acid tungstate (Folin-Wu), and varying quantities of equivalent 0.15 M $Ba(OH)_2$ and 0.15 M $ZnSO_4$. Similarly recovery experiments using the above filtrates were done. The entire set of filtrates was analyzed both by a standard Cu^{II} reduction method and glucose oxidase. The enzyme method resulted in a standard deviation between filtrates of 5 mgm% and a 97% mean recovery of added glucose. In the Cu^{II} reduction method the standard deviation between filtrates was 20 mgm% and the mean recovery 114%.

Apparently the glucose oxidase method is relatively free of filtrate effect. In all reduction methods the concentration of reducing substances is dependent upon the type of filtrate. This preliminary observation may infer that the enzyme is specific for glucose. The observed standard deviation between filtrates is greater than that obtained on the synthetic control serum. A possible explanation is that small quantities of either Cu^{II} and Wo^{III} were in the filtrates. Also certain filtrates as the Folin-Wu do not remove uric acid. Both factors tend to inhibit the enzyme. A larger study to evaluate filtrate effect and enzyme specificity is planned. The glucose in a filtrate is to be isolated chromatographically and then analyzed quantitatively. The concentration then would be compared with the result from a heterogenous filtrate.

Accepting the Conway micro-diffusion technique for analyzing blood urea nitrogen as a reference, the monoxime method gives results that are statistically comparable.

Summary

A micro-chemical method is presented for simultaneously analyzing blood glucose and urea nitrogen on a single sample of either blood, serum, or plasma. The only micro-measurement necessary is the measurement of the sample. The procedures utilize the glucose oxidase and diacetyl monoxime methods respectively for glucose and urea nitrogen.

The method is rapid, uses simple equipment, and has a range of error commonly experienced in biological work. The normal values fall within

a range commonly observed in clinical medicine.

It is proposed that the enzyme method is specific for glucose and free of the filtrate variables observed in Cu^{II} reduction methods.

Using the Conway micro-diffusion technique as a reference, the monoxime method for urea nitrogen gives statistically comparable results.

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ADDENDUM

Kingsley G. R. and Getchell G., Clin. Chem., 6:466-425, 1960 reported their experience doing direct analyses for glucose on 20 microliter samples of plasma. Their method utilized the addition of sulfuric acid and shifting the maximum absorbance of the o-dianisidine from 400 mu to 525 mu. Since many colorimeters are relatively insensitive at 400 mu, this shift is particularly useful.

The procedure for glucose analysis is modified by the addition of 3.0 ml of 25% H_2SO_4 (v/v) instead of 1 drop of concentrated HCl to the reaction mixture at the end of thirty minutes.

The ensuing pink color is stable for at least 10 hours. With a glucose concentration of greater than 230 mgm%, the calibration curve becomes non-linear. This modification compares statistically with the techniques described in this paper.

MICROSCOPICAL EXAMINATION OF THE STOOL FOR STEATORRHEA

A simple, practical and valid method for the screening of random stools for evidence of malabsorption, particularly of fat, is presented.

Small, representative amounts of a thoroughly mixed specimen, consisting of lumps or pools about 5 mm. in diameter, were placed on each of two slides.

Neutral Fat. Two drops of water were added to the stool sample on one of the slides and thoroughly mixed with an applicator stick. Two drops of 95% ethyl alcohol were added and mixed. Several drops of a saturated solution of Sudan III in 95% ethyl alcohol were added and mixed with the edge of a coverglass before the latter was applied. The preparation was then examined microscopically (430X) for yellow or pale orange refractile globules, special attention being paid to the edges of the coverglass since neutral fat tends to float there.

Split Fat. Soaps and other fat combinations are dissociated as free fatty acids by the addition of several drops of 36% acetic acid to the stool on the second slide and mixing to a homogenous consistency. Several drops of Sudan III solution were added and the preparation mixed with a coverglass which was then applied. The slide was *gently* heated over an alcohol burner until it began to boil. The process was quickly repeated two or three times to melt the fatty acid crystals. The preparation was examined while still warm (magnification 430X). The stained free fatty acids appear when warm as deep orange fat globules from which spicules and soap forms resembling the pinna of the ear crystallize as the preparation cools. A micrometer eyepiece was used to determine the diameter of the fat globules.

The general appearance of the stool may divulge the fatty content.

A very few, if any, neutral fat globules are found in the normal stool. Stools from some patients with pancreatic disease show a marked increase of neutral fat. In most patients with steatorrhea from other causes, there is no increase in neutral fat and without the addition of acetic acid the excessive fat will not be shown. Large amounts of neutral fats may be due to the ingestion of mineral oil, castor oil or low-calory mayonnaise, the use of rectal suppositories and other such contaminants.

Microscopic reading of split fat was based on the following criteria:

Normal—Up to 100 globules per high power field, 1-4 μ in diameter; much fecal material.

Slightly increased—100 or more globules per high power field, 1-8 μ in diameter; less fecal material.

Definitely increased—Over 100 large globules per high power field, 6-75 μ in diameter; no fecal material in background.

The relative accuracy of this procedure was determined by comparing the results with chemical analyses. Drummey, G. D., Benson, J. A., Jr., and Jones, C. M., Department of Medicine, Harvard Medical School, and the Medical Services, Massachusetts General Hospital, Boston, Massachusetts. *The New England Journal of Medicine*, 264, 2, 1961, pp. 85-87.

A SOURCE OF ERROR IN ELECTRONIC BLOOD CELL COUNTS

It would appear that when very large platelets are present in sufficiently large numbers, and when megakaryocyte fragments and gross clumping are present, automatic cell counting methods are subject to a considerable degree of error.

One patient had a platelet count of 3,000,000 per cu. mm. with many as large as 8 μ to 9 μ in diameter. White blood cell counts of this patient as determined by a Coulter counter were as much as 150% higher than hemocytometer counts. The red corpuscle counts were also higher as determined by the Coulter counter. While a difference in count of 380,000 was demonstrated, the percentage error was much less in counting the red corpuscles. Eaton, A. G., University of Alberta Hospital, Edmonton, Alberta, Canada. *The Journal of Medical Laboratory Technology*, 18, 1, 1961, pp. 41-42.

CALCULATION OF SERUM TOTAL PROTEIN FROM THE INTEGRATOR TRACE OF SCANNED PAPER ELECTROPHORESIS STRIPS AND COMPARISON WITH A CHEMICAL METHOD*

RICHARD E. BUDD, *Chief Hospital Corpsman, U. S. Navy Clinical Laboratory Technician in RADM G. W. Calver's Physical Chemistry Research Laboratory at U. S. Naval Medical School, National Naval Medical Center, Bethesda, Maryland.*

Quantitative results of serum protein electrophoretic patterns are obtained in this laboratory by scanning with an automatic recording densitometer (Analytrol). Results are calculated and reported in percentage distribution of each component. A total protein determination is performed on each serum by the biuret method.[3] This is reported in Gms% along with the percent distribution of each protein fraction.

Although the total area shown on a scanning does not provide a good measure of total protein concentration[1] it was felt that an estimate could be made from the tracing, using the integrator tooth count ("blips") with reasonable accuracy.

A method of calculation of total protein using the integrator trace representing the total area beneath the curve and comparison with a chemical (biuret) method is presented.

Materials and Methods

It was considered that the best approach to calculating total protein concentration using the integrator trace would be to obtain a factor (F) which when multiplied by the total "blip" count, representing the total area under the curve, would give the total protein in Gms%.

A formula for this was:

$$F = \frac{X}{A} \qquad \begin{array}{l} X = \text{Total protein (biuret)} \\ A = \text{Total "blips"} \end{array}$$

This gave a factor for each strip. Since protein patterns are run in duplicate the total "blip" count was averaged and a new factor calculated:

$$F' = \frac{X}{A'} \qquad A' = \text{average total "blips"}$$

After the new factor (F′) was determined it was found that the same factor could not be used for all the tracings as the "blip" count varied with the concentration of protein represented by the tracing (Fig. 1).

The majority of the patterns resulted in total counts in the range of 120 to 300 with rare exceptions. This range was broken up into segments of 30 counts and factors (F′) calculated in 40 cases comprising about 5 patterns with average "blip" counts in each of the various ranges. These factors were averaged (Table 1).

TABLE 1
Factors for calculating total protein from "blip" counts in 8 different ranges.

"Blip" Count	Less Than 120	121–150	151–180	181–210	211–240	241–270	271–300	Over 300
Factor.....	.050	.040	.037	.035	.033	.031	.028	.025

*The opinions expressed in this article are the private ones of the author and not to be construed as official or reflecting views of the Navy Department.

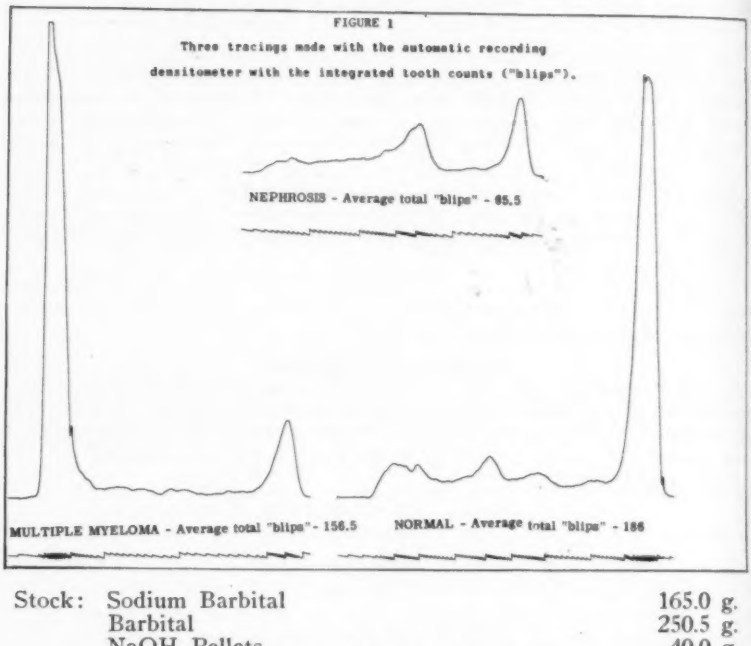
*Received for publication January 1961.

Electrophoresis:

Electrophoretic separation and staining was carried out on 99 patients by the method described in Spinco Model RB Instruction Manual[1] with a minor modification. This modification consisted mainly of preparing our own Veronal buffer pH 8.6, ionic strength 0.075:

FIGURE 1

Three tracings made with the automatic recording densitometer with the integrated tooth counts ("blips").



Stock:	Sodium Barbital	165.0 g.
	Barbital	250.5 g.
	NaOH Pellets	40.0 g.
	Dissolve and dilute to volume with distilled water to 20 L.	

Working: Dilute 750 ml. of stock solution to 1 L.

Protein:

Total protein concentration was determined by the biuret method.[3]

The formula for calculated total protein is expressed as follows:

(Average total "blips") X (F′) = Total protein

The average total "blips" is derived from the tooth count of the integrator tracings of the scanned patterns in duplicate.

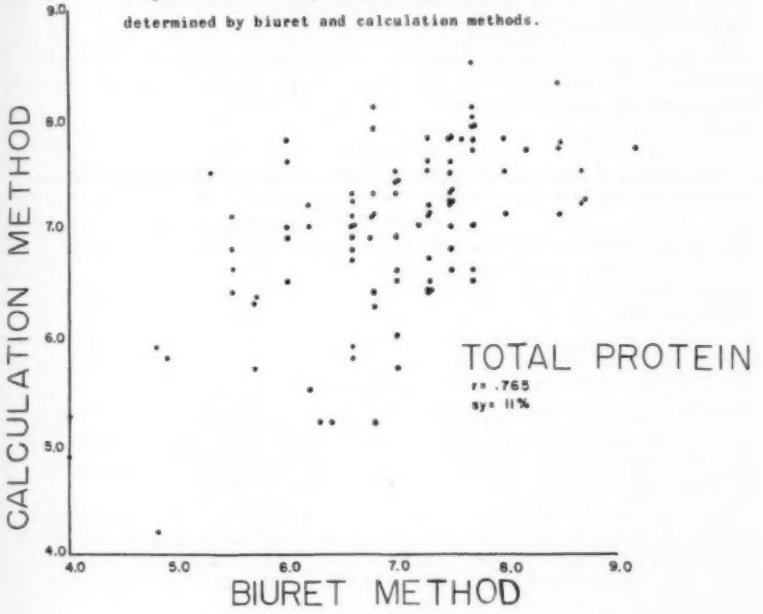
The calculated total protein concentrations using the factors in Table I were plotted against comparative findings obtained by the biuret method in the same specimen (Fig. 2).

The significance of the correlation in the resultant plot was tested by Pearson's product-moment correlation coefficient[4]: $rxy = \frac{\Sigma(xy)}{N\sigma x\sigma y}$ where x and y are the deviations of the observed values from the respective means, N is the total number of cases, and σx and σy are the standard deviations of the respective means. With this calculation, an r of 1.0

represents perfect correlation whereas an r of 0 indicates no correlation. The standard error of estimate of the calculated determinations (the biuret results considered as reference) was tested according to the following formula[2]: $Sy = \sigma x \sqrt{1-r^2}$ where σx equals the standard deviation as above and r is the correlation coefficient. For purposes of comparison the standard error of estimate is expressed in percentage of the mean.

FIGURE 2

Comparison of total protein concentration as determined by biuret and calculation methods.



Results

The mean total protein concentration by the calculated method and biuret method was found to be 7.0 gms% for both.

The correlation is fair, r being .765 and the standard error of estimate 11.0 percent at the mean.

The percent differences between the calculated total protein concentrations and those obtained by the biuret method were determined and the mean percent difference was found to be 9.2 percent.

In 23 cases with abnormal total protein (from the 99 cases studied), normal range being 6.0 to 8.0 gms%, the mean percent difference was found to be 13.9 percent.

Discussion

The coefficient of correlation, r, being .765 indicates approximately 77% correlation (as against 100% or perfect correlation).

It follows that the calculation method is suitable as an estimate of the total protein concentration within normal ranges but due to the difference in the abnormal ranges the method is not suitable in all cases as a standard procedure. The abnormal concentration of total protein is usually apparent from the scannings of paper strips (fig. 1).

Three tracings made with the automatic recording densitometer are shown in Figure 1. This demonstrates a normal pattern and the abnormal ranges sometimes encountered. The total protein concentration in the three cases by the biuret method and by the calculation method are shown in Table 2.

Extreme care must be made in measuring the amount of sample (6 lambda) and application to the paper strip with the striper to avoid any loss of the specimen.

TABLE 2

Comparison of calculated T.P. and biuret method in normal and abnormal ranges.

Case	Biuret T.P. Gms %	Average Total "Blips"	Factor	Calculated T.P. Gms %
Normal	7.0	186	.035	6.5
Multiple Myeloma	15.6	156.5	.037	5.8
Nephrosis	5.4	65.5	.050	3.3

Summary

A formula is given for estimating the total protein concentration directly from the tracing of the scanned strip using the integrated tooth counts ("blips") and a factor. This factor was arrived at by analysis of 40 cases with average "blip" counts in 8 different ranges.

The results of total protein concentration (99 cases) evaluated by a statistical correlation between a chemical (biuret) method and a method of calculation from the tracing of scanned paper electrophoresis strips show fair correlation. The correlation coefficient (r) was .765 and the standard error of estimate 11.0% at the mean.

With this fair correlation it is felt that an estimate can be made of the total protein concentration from the tracing of the scanned paper electrophoresis strips in most cases. The exceptions will be in the extreme low and high ranges which will usually show up as an abnormal tracing.

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A SIMPLIFIED METHOD FOR DETERMINATION OF PROSTATIC ACID PHOSPHATASE USING BODANSKY UNITS

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Abdul-Fadl and King[1] reported the inactivation of prostate acid phosphatase by 0.01 M L-tartrate. Since then, Fishman and Lerner[3] and Stolbach, Nisselbaum and Fishman[4] have devised methods for the determination of prostatic acid phosphatase. Their results were reported in comparable Gutman units. However, many laboratories prefer the method of Bodansky[2] or its modifications and report results in Bodansky units. The method that follows allows these laboratories to report "prostatic acid phosphatase" activity in Bodansky units. It has been used successfully in this laboratory.

Reagents: Stock glycerophosphate buffer. 10 GM sodium-beta glycerophosphate and 8.48 GM sodium barbital are dissolved in 1 L of distilled water. Layer with 10 ml of petroleum ether. Keep in refrigerator.

Acid phosphatase substrate. 250 ml of stock buffer. 25 ml of 0.1 N acetic acid diluted to 500 ml. with distilled water. Adjust to pH 4.5. Add 7.5 ml. of petroleum ether.

Acid phosphatase tartrate substrate. 250 ml. of stock buffer 25 ml. of 0.1N acetic acid and 25 ml. of 0.2 M L-tartrate solution. Dilute to 500 ml. with distilled water. Adjust to PH 4.5. Add 7.5 ml. of petroleum ether.

IN acetic acid. 5.7 glacial acetic acid diluted to 100 ml. with distilled water.

0.2M L-tartrate solution. Dissolve 3.002 GM tartaric acid in 50 ml. distilled water. Add 35 ml. of IN sodium hydroxide. Adjust to PH 4.5 with IN sodium hydroxide or IN hydrochloric acid. Dilute to 100 ml. Store in glass stoppered bottle in refrigerator.

Molybdic acid solution. Dissolve 12.5 GM ammonium molybdate in 150 ml. of water. To this add a solution of 42 ml. of conc. sulfuric acid in 150 ml. of distilled water, previously cooled. Dilute to 500 ml. with distilled water.

Phosphorous standard. Dissolve 0.3515 GM KH_2PO_4 (anhydrous) in 500 ml. distilled water. Add 10 ml. of ION H_2SO_4. Dilute to 1 L with distilled water (1 ml. = .08 mg. P).

Aminonaphthol sulfonic acid reagent. To 0.5 gm. pure 1,2,4-aminonaphthol sulfonic acid add 195 ml. of 15% $NaHSO_3$ solution and 5 ml. of 20% sodium sulfite solution. Shake until dissolved. Keeps four weeks in refrigerator.

METHOD

Procedure:

Into 6 x 150 mm. test tubes, place—	1.	2.	3.	4.
Acid phosphatase substrate pH 4.5	9.0 ml.	9.0 ml		
Acid phosphatase tartrate substrate			9.0 ml.	9.0 ml.
Warm in 37° C water bath, 5-10 minutes				
Serum (unhemolyzed)	1.0 ml.	1.0 ml.	1.0 ml.	1.0 ml.
30% trichloracetic acid		2.0 ml.		2.0 ml.
Incubate in 37° C water bath exactly one hour. Remove.				
30% trichloracetic acid	2.0 ml.		2.0 ml.	
Mix. Let stand 5-10 minutes. Centrifuge 10 min. at 2500 RPM.				

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Prepare a Standard: 1.0 ml. of phosphorous standard (1 ml. = .08 mg. P) into a similar test tube, add 9 ml. of distilled water and 2 ml. of 30% trichloracetic acid. Mix.

Prepare a Blank: 10 ml. of distilled water and 2 ml. of 30% trichloracetic acid. Mix.

Remove 5.0 ml. of each supernate and place in the properly labeled tubes, add 3.6 ml. of distilled water and 1 ml. of molybdic acid solution to each. Mix and add 0.4 ml. of aminonaphthol sulfonic acid to each. Mix. Let stand 8 minutes.

Read all tubes at 520 mu (or 660 mu) in a spectrophotometer, setting the blank at 100% T (zero density).

CALCULATIONS

A. $\frac{D_1 - D_2}{D \text{ stand}} \times 8 =$ acid phosphatase in Bodansky units/100 ml.

B. $\frac{D_3 - D^4}{D \text{ stand}} \times 8 =$ acid phosphatase Bodansky units in tartrate inhibited test.

A — B = "prostatic acid" and phosphatase in Bodansky units/100 ml. serum.

DISCUSSION

This test was performed mostly on the patients of a hospital in a 40 to 60 age group. The normal range for those patients for acid phosphatase was found to be 0.0 to 1.0 Bodansky units with a prostatic acid phosphatase of 0.0 to 0.6 units.

In case of proven carcinoma of prostate, the acid phosphatase values were all over 2.0 Bodansky units. The acid phosphatase activity in these sera, after tartrate inhibition, was never higher than 0.6 Bodansky units. Satisfactory results have been obtained using half quantities of serum and reagents. A suitable micro-method could be devised.

SUMMARY

A simple method for prostatic acid phosphatase is presented which may be reported in Bodansky units.

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A NEW RAPID METHOD FOR DETECTING THE D^u FACTOR (a)*

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Race, Sanger and Lawler[6] have defined the D^u factor as follows: "If the sample of cells gives some, but not all, of the reactions expected of D when tested by the various methods against the battery of agglutinating and incomplete anti-D sera, we call it D^u." It is therefore obvious that the D^u factor frequently is not discovered during routine D typing, and such bloods are considered D negative. Past studies have shown that a D negative individual transfused with D^u positive blood may become sensitized by those cells, producing an anti-D antibody. Therefore, in the blood bank a major problem is the elimination of D^u sensitization by transfusion of D^u positive blood. Some Centers are meeting this problem by doing an indirect antiglobulin test[1] on all D negative bloods found in routine anti-D testing. Because it is time consuming and expensive to perform this excellent test, many centers have found it not to be a practical approach.

The purpose of this report is two-fold: (1) to determine the incidence of the D^u factor in our population so that the magnitude of the problem of D^u sensitization may better be evaluated, and (2) to describe a new, rapid method using bromelin for detecting the D^u factor.

Materials and Methods

A. Bromelin Stock Solution[3,5]:
   1. 0.5% bromelin powder dissolved in nine parts isotonic saline and one part Sorenson phosphate buffer, (0.15 M), pH 5.5.
   2. 0.1% sodium azide and 0.05% actidione (Upjohn) added as a preservative.
   3. Store at 4° C. Discard after one month or if fungus or black discoloration appears.
   4. Centrifuge before use and use supernatant solution.

B. Diluted anti-D serum:
   1. One part commercial anti-D serum.
   2. Two parts 0.85% saline.

C. Bromelin D^u Method:
   1. Into a 10 x 75 mm tube place two drops 0.85% saline.
   2. Add one stick of cells to be tested, making a 4-5% suspension of cells.
   3. Add two drops bromelin solution.
   4. Incubate 10 minutes at 37° C.
   5. Wash once in multiple volumes of 0.85% saline.
   6. Decant and add two drops diluted anti-D serum.
   7. Centrifuge immediately one minute at 1000 R.P.M.
   8. Examine macroscopically for agglutination by gently tapping the bottom of the tube to dislodge the cell button.

D. PVP anti-D Method[2]:
   1. PVP Mixture:
      a. 5 cc commercial anti-D testing serum
      b. 10 cc 0.85% saline
      c. 15 cc 10% PVP
   2. Place in a 10 x 75 mm tube two drops PVP mixture.

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3. Add one stick of cells to make a 4-5% suspension of cells.
4. Incubate at room temperature for five minutes.
5. Centrifuge for five minutes at 2000 R.P.M.
6. Shake tubes for 30 seconds on a Kahn shaker.
7. Read macroscopically for agglutination.

All blood specimens tested were obtained from volunteer donors appearing at our Blood Center. The only selection was in the inherent motivation of a volunteer donor population. The antisera used in this study were commercially obtained from three sources and met the minimum requirements of the National Institutes of Health.

For the D^u incidence portion of this study, all blood specimens were tested for D as outlined by the producer of the antisera. D negative specimens were further tested for the C and E factors, using anti-CD and anti-DE sera respectively. The tube indirect antiglobulin test[1] with a one hour incubation time was done on every D negative blood, in order to determine the presence of the D^u factor.

Only D negative blood specimens, as determined by the PVP anti-D method, were used in the testing of the new bromelin procedure. Using two different anti-D sera, all such negative D were tested in duplicate by both the bromelin and indirect antiglobulin methods for the determination of D^u.

Results

9013 blood specimens were tested to determine the incidence of the D^u factor in our population. After D typing with the method as outlined by the commercial source of antisera, all blood specimens found to be D negative were further tested by the indirect antiglobulin method. Those specimens which presented positive agglutination by this test were presumed to have the D^u factor. 50, or 0.55%, such bloods were encountered. All D negative specimens were also tested for the C and E factors. The incidence of the D^u factor can be seen from Table 1. These results compare favorably with other published incidence studies. From our results of incidence of D^u, it can be seen that if D^u typing is not done on all D negative donor bloods, the danger of D^u sensitization may occur in 0.55% of D negative blood transfusions. Therefore, we feel that all D negative donor bloods should be tested for the D^u factor. For this reason we developed the new bromelin D^u method in order to have a rapid, reliable method for testing for D^u.

TABLE 1

Incidence and Distribution of D^u Factor after D Typing by the Method as Outlined by the Commercial Source.

Typing	Number	Percent	D^u POSITIVE	
			Number	Percent
D+	7288	80.87		
D−	1725	19.13		
dCe	93	1.03	23	24.7
dcE	74	0.84	13	18.9
dCe	16	0.17	11	87.5
dce	1541	17.10	3	0.19
Total	9013	100	50	

TABLE 2

Comparative Sensitivity of the Bromelin and Antiglobulin Techniques in Demonstrating the D^u Factor

		ANTI D SERA REACTIONS															
		Tests	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
		Bromelin	−	−	+	+	+	+	+	−	+	−	+	−	+	+	−	−
Typing	Number	Antiglobulin	−	−	+	+	+	−	+	+	+	−	−	−	−	−	+	+
dCe	186				131		136		10		3		0		5		1	
dcE	156				129		14		10		0		0		2		1	
dCE	18				7		7		3		0		0		0		0	
dce	832				831		0		0		0		1		0		0	
Total	1192				1098		57		23		3		1		7		2	

The reliability of the new bromelin-D^u procedure was evaluated as seen in Table 2. 1192 blood specimens found to be D negative with the PVP anti-D procedure were tested for the D^u factor by the bromelin and the antiglobulin procedures. Using two different anti-D sera in each test system, all specimens were studied in duplicate. 1098 were found to be D^u negative and 57 D^u positive with both anti-D sera and by both methods. One was positive with one antisera by both methods. 23 were positive with both antisera by the bromelin D^u method, and with one antiserum by the antiglobulin method. Three were postive with one antiserum by bromelin and with both antisera by the antiglobulin method. Nine gave equivocal results with the bromelin method. Seven of these were positive with both antisera by the bromelin D^u test, and negative with both antisera by the antiglobulin method; the other two were positive to one antisera by bromelin and negative by the antiglobulin method to both antisera. Whether these reactions were false positives by bromelin or weak D^u samples was not determined. One other specimen gave equivocal results in that it was positive to both antisera by the antiglobulin method and negative to both antisera by bromelin. Again in this case whether it was a false positive reaction by the antiglobulin method or a weak D^u undetected by the bromelin method was not determined.

Discussion

In 1945, Wiener[10] observed some bloods that were clumped weakly by using ordinary Rh typing serums, which he called Rh variants. Stratton,[9] in 1946, described a variety of the D factor which he called D^u, after the nomenclature of Race and co-workers.[6] He further postulated that this factor was sited at the Dd locus, and adopted the term D^u to designate a number of variants of D comprising over 1% of all D negative bloods. Ruffie and Carriere[8] showed that a D negative individual could produce an antibody against D when he received D^u cells by transfusion. A more complete picture was obtained with the demonstration that very rarely D^u positive individuals could develop anti-D after being transfused with D positive bloods. Accordingly, the following approach is resorted to in transfusion therapy: D^u bloods from a donor should be considered D positive, and only transfused into D positive individuals; a blood recipient who is D^u positive should be considered as a D negative, and receive D negative blood for transfusion.

TABLE 3

Incidence and Distribution of D^u Factor in New York Caucasians

Typing	Number	D^u POSITIVE	
		Number	Percent
Cde/cde	161	72	44
cdE/cde	92	19	20.7
cde/cde	628	3	0.5
Total:	881	94	

The major problem that D^u blood introduces into transfusion therapy is the possibility of such bloods being typed D negative and sensitizing D negative recipients. That the problem is serious can be seen in Table 3, summarizing the D^u distribution of Caucasian donors in New York, as performed by Rosenfield et al.[7] The present study indicates a parallel but less common distribution of D^u in our population, a predominantly Caucasian group of Scandinavian ancestry (Table 1). The use of the PVP anti-D or bromelin anti-D methods[4] for D typing of donors appears to demonstrate some D^u bloods in routine typing, leading to the classification of D positive, and assuring their transfusion use to D positive individuals. However, many blood banks use neither of these methods, and so, as seen in Table 1, the problem has not been alleviated with use of the method recommended by the commercial source of anti-D sera. A blood center may minimize this risk of transfusing D^u blood into D negative individuals by using only cde/cde bloods. This appears wasteful and involves a risk of 0.19% of these bloods being D^u positive in a Caucasian population. A sound approach would be further testing of all D negative typings by the indirect antiglobulin procedure for D^u. However, this procedure is time consuming and appears economically unsound in the large Blood Center. For these reasons, the bromelin technique was modified in an attempt to provide a practical method for the demonstration of D^u bloods.

Comparison of the bromelin technique with the antiglobulin procedure rendered essentially similar results. One specimen gave a positive result with the antiglobulin test and a negative result with the bromelin test, and only three specimens gave positive antiglobulin reactions with both antisera and positive bromelin reactions with one antiserum. Nine specimens gave positive bromelin results with one or both antisera and negative antiglobulin reactions with both antisera. Whether this reaction is due to a false positive bromelin reaction, or weak D^u bloods are being demonstrated, has still to be determined. However, it would appear probable that weak D^u bloods are being demonstrated by the bromelin technique, because 23 specimens showed positive reactions to both antisera by the bromelin method and positive reactions to one antiserum by the antiglobulin method, as compared to only three showing the reverse reactions.

A few words of caution with the use of bromelin appears indicated. The bromelin reagent may be very sensitive to cold agglutinins. Therefore, the presence of cold agglutinins in the anti-D sera may lead to false positive reactions. Such cold agglutinins can be demonstrated by the

Reported by Rosenfield et al,[7] 1951.

disappearance of agglutination, after heating a few minutes in a 37° C water bath. The bromelin procedure is frequently very sensitive in demonstrating weak erythrocyte antibodies. The anti-D sera used may contain antibodies other than anti-D that are absorbed out, or are so weak as not to lead to positive reactions when used according to the manufacturers' recommendations. However, when such anti-D sera are used in a bromelin procedure, false positive D^u results may be obtained. The dilution of commercial anti-D sera 1:3 with isotonic saline is not only economically desirable, but essential in the bromelin test. Prior studies have indicated that high concentrations of albumin may lead to false positive reactions.[4] Most commercial antisera have sufficient albumin added to make false positive bromelin reactions a distinct possibility.

It should be emphasized that the D^u problem is primarily the concern of blood banks which type donors. No advantage, and possible harm, may result from the extensive typing of blood recipients for the D^u factor. If such recipient typing is done, the D^u recipient should receive D negative blood.

Summary

The problem that D^u bloods present to a Blood Center was discussed, and population data for this factor was presented. Also presented was the bromelin D^u test. A comparison of the bromelin and the antiglobulin methods was shown and discussed. The new bromelin test appeared to compare favorably in sensitivity, and may be even more sensitive in detecting low grade D^u, although this has still to be determined.

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THE ERRONEOUS DIAGNOSIS OF PROTEINURIA DUE TO BACTERIAL CONTAMINATION

Sörensen has shown that in the presence of protein many indicators fail to develop the color appropriate to the pH of the solution under test. This phenomenon, called "the protein error of indicators", has been utilized in testing for proteinuria.

A simple application of this test—marketed under the name of *Albustix*—consists of a paper strip, one end of which is impregnated with a citrate buffer which maintains the pH at about 3, and an indicator, tetrabromphenol blue. When dipped into a fluid containing proteins, the strip changes color from yellow, through green to blue, the final color depending on the concentration of protein present. The physiological range of urine pH has negligible effect on the result of the test.

Since urine specimens contaminated with *Proteus* and strongly alkaline have been known to give a false positive *Albustix* test, this type of contamination was the subject of the present investigation.

After studying the effect of this bacterial contamination, it was concluded that false positives of this kind are of little practical significance, since such a false positive occurs only after a urine specimen has stood at room temperature for three days.

It was noticed that whenever false positive results due to *Proteus* contamination were obtained, the strip effervesced when dipped into the urine and the color change produced was patchy.

Occasional false positive results with *Albustix*— probably due to excessive alkalinity of the urine—have been reported, with some authors noting that false positive results could be obtained on the urine of subjects who had ingested alkali and restricted their fluid intake.

One instance of a false positive has been recorded in a heavily infected urine 24 hours after it had been passed, although the identity of the contaminating organism was not stated. The pH was 6.4. Huntsman, R. G. and Liddel, J., *Guy's Hospital Reports*, 109, 3 1960, pp. 179-183.

NEOPLASTIC LYMPHOID RETICULUM CELLS IN THE PERIPHERAL BLOOD: A HISTOCHEMICAL STUDY

Lymphoid organs (lymph nodes, spleen, etc.) may be said to contain at least two types of leukocytes—lymphocytes and reticulum cells. In rare cases, where there is neoplastic proliferation of the reticulum cell system, some of the neoplastic cells may be found in the peripheral blood, sometimes in large numbers (known as reticulum cell leukemia or leukemic reticuloendotheliosis). The existence or identity of these cells still are not universally accepted. These cells have often been confused with cells of the lymphoid series, especially of the atypical variety.

On the average they are 1½-2½ times the size of lymphocytes, round or slightly oval. In most the cytoplasmic edge is irregular and small pseudopod-like projections are commonly seen (3 photographs are included). With Wright-Giemsa stain, the cytoplasm stains blue or grayish-blue and in some of the cells several azurophil granulations are present. These are heavier than the fine granulations of monocytes. The nuclei are round or slightly oval and approximately ½ the size of the cell, in contradistinction to the monocytes with their indented or reniform nucleus filling usually 7/10 of the cell. Frequently the nucleus is located eccentrically. The nuclear membrane is thick and unusually distinct. The chromatin is spongy, heavy in appearance, less clumped than the chromatin of lymphocytes, but not so fine as the chromatin of the blast cell. It is distinctly different from the fine but loosely woven network of monocytic chromatin. Many of the cells show indistinct nucleoli; only rarely a distinct nucleolus is present. Occasionally elongated forms with tapering ends are seen.

Study by cytochemical methods and phase microscopy showed them to be very similar in their reactions to the "fixed" or tissue reticulum cells and in many respects to monocytes, but markedly different from cells of the lymphocytic system, Mitus, W. J., Mednicoff, I. B., Wittels, B., and Dameshek, W., Blood Research Laboratory, the Pratt Diagnostic Clinic-New England Center Hospital and the Department of Medicine, Tufts University School of Medicine, Boston, Massachusetts, *Blood*, 17, 2 1961, pp. 206-215.

EVALUATION OF THE ANTI-STAPHYLOCOCCAL ACTIVITY OF FABRIC TREATED WITH GERMICIDES

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Introduction

The emergency of hospital-acquired staphylococcus infection as a serious public health problem has demanded control over sanitation in all areas in the hospital environment which might play a role in staphylococcus transmission. Hospital linens constitute one such area. Good laundering practice produces linens out of the wash wheel which are free of staphylococci,[3,4] but the fabric is subject to contamination from the time it leaves the wash wheel until it is returned to the laundry as a soiled material for re-washing.[2] Fabric contaminated with organic material, such as food or body fluids, may become a focus of staphylococcus growth; a potential threat to the patient and the hospital environment is thus posed.

In recent years a number of products which are designed to effect an impregnation of fabric with anti-bacterial agents have been merchandised. These materials are usually added during the last rinse in the laundering cycle, but at least one product incorporates a germicidal system in laundry soap. In theory, the presence of an effective anti-bacterial system protects the fabric against becoming a focus of bacterial growth.

The clinical bacteriologist is frequently called upon to determine the efficacy of such products, either as an aid to the hospital purchasing department or as a control over their proper utilization in the hospital laundry.

The accepted procedure for evaluating the anti-bacterial activity of fabric is the agar plate method of Schwartz *et al.*[5] The method involves placing the test fabric on the surface of a poured plate of seeded F.D.A. agar. After incubation, anti-bacterial activity is manifested by a zone of inhibited growth around the test fabric. Removal of the cloth allows observation of inhibition in the contact area between the cloth and the agar.

The present work describes a modified test procedure, the Contact Plate Method, for evaluating anti-bacterial activity in fabric. This test appears to give a more precise evaluation of anti-staphylococcal activity than the Agar Plate Method.

MATERIALS AND METHODS

Cultures Used: A mixture of five coagulase positive strains of *Staphylococcus aureus* was used in all studies. Each of the organisms was a recent isolate from a case of clinical staphylococcus infection. The individual cultures were grown separately in brain heart infusion broth for 24 hrs. at 37°C. The test mixture was prepared by combining equal volumes of the five cultures.

Agar Plate Method: The method Schwartz *et al*[4] (American Association of Textile Chemists and Colorists Test-AATCC) was followed. A

[a] Received for publication December 1960

tube containing 15 ml. of F.D.A. agar was melted and cooled to 45°C, inoculated with 0.1 ml. of the staphylococcus mixture and poured into a sterile Petri dish. The test fabric (1 sq. in.) was pressed into intimate contact with the surface of the seeded agar. Specimens were incubated 24 hrs., at 37°C. The zone of inhibition around the cloth was measured. The fabric was then removed with forceps and the contact area under the cloth was observed for growth.

Two Hour AATCC Method: The procedure of Schwartz[5] *et al* includes an optional modification in samples giving questionable results: the cloth specimen was removed after 2 hrs. incubation, after which the Petri plate from which the sample had been removed was incubated an additional 22 hrs. The agar was then observed for growth in the areas surrounding and under the cloth imprint.

The Contact Plate Method. The surface of a tellurite glycine agar plate was inoculated with 0.1 ml. of a 1 to 100 water dilution of the staphylococcus mixture. The inoculum was spread evenly over the entire surface of the agar with a sterile glass rod bent in a "hockey stick" shape. The test cloth was then pressed firmly against the surface of the agar. Test plates were incubated for 24 hrs. at 37°C. The zone around the cloth was observed for growth as was the area of contact between the fabric and the agar.

RESULTS

1. Comparative Evaluation of Cloth with the Three Test Methods.

Six different commercial preparations were tested under controlled laboratory conditions using cotton diapers washed in a home-type washing machine. Five of these agents were quaternary compounds used by addition during the last rinse in the washing cycle. The fifth was a soap containing anti-bacterial agents. All products were used according to the manufacturers' directions. The washed and dried fabrics were tested for activity against staphylococci by the three methods described above.

Five of the six test fabrics showed complete inhibition of growth at the interface between the cloth and the agar surface, with a zone of inhibition around the cloth. The zone of inhibition was broader with the Contact Plate Method (average 6.2 mm) than with either the standard AATCC (average 3 mm) or the 2 hr. modification of the AATCC test (average 2.6 mm). Further, the inhibition of growth under the test fabric was more evident with the Contact Plate procedure than in the other two test methods. The black staphylococcus growth on tellurite glycine agar afforded a contrast which facilitated reading of the test results.

The sixth fabric showed complete inhibition at the cloth-agar interface but no zone in the Contact Plate Test. The same sample, using the two AATCC procedures, yielded plates showing no zone of inhibition, and it was not possible to determine with certainty whether growth had occurred under the fabric.

Figure 1 shows representative results. It will be noted that with test fabric 1, a zone of inhibition is clearly evident with each of the methods; whereas with test fabrics 2 and 3 no zone was apparent in any of the

tests. Of greatest importance is the difficulty in determining the presence or absence of growth at the cloth-agar interface with the two AATCC procedures.

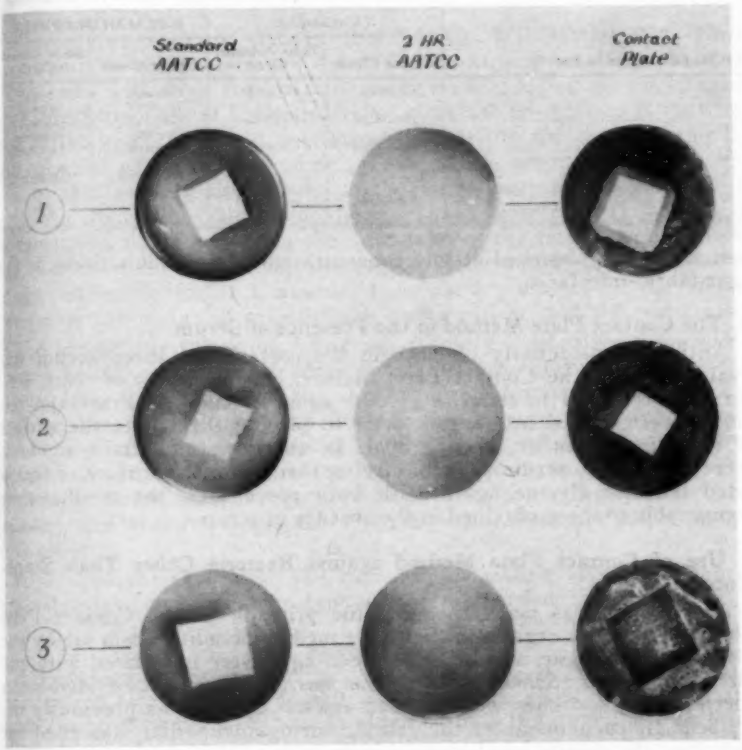

Figure 1.
Comparison of Three Evaluation Procedures on Germicide Treated Fabric.
Samples 1 and 2 treated with commercial germicides during laundering.
Sample 3 received no treatment other than laundering.

2. The Significance of the Zone of Inhibition

It is well known that the production of a zone of inhibition requires that the inhibitory material be diffusible in agar. The data in Table 1 illustrate that the production of a zone is a function of both the germicide and its concentration. With Vancide-26* inhibition at the cloth-agar interface occurred at low concentrations with an absence of a zone around the fabric, whereas at higher levels of Vancide-26, both inhibition under and around the test swatch occurred. With hexachlorophene** the opposite result was noted—a zone of inhibition around the

** 2,2' methylenebis hexachlorodiphenylmethane, manufactured by Sindar Corp.

TABLE 1
Contact Plate Method: Response of Staphylococci to Hexachlorophene and Vancide-26

P.P.M. Germicide in Fabric	STRAPHYLOCOCCUS AUREUS GROWTH RESPONSE			
	VANCIDE-26		HEXACHLOROPHENE	
	Under Cloth	Zone Around Cloth	Under Cloth	Zone Around Cloth
0	Growth	Growth	Growth	Growth
1	.....	.....	Growth	Growth
2	Inhibited	Growth	Growth	Growth
3	.....	.....	Growth	Inhibited
4	Inhibited	Growth	Growth	Inhibited
5	.....	.....	.....	.....
6	.....	.....	Inhibited	Inhibited
7	.....	.....	.....	.....
8	Inhibited	Inhibited	.....	.....

test cloth was observed at low concentrations, but no inhibition at the agar-fabric interface.

3. The Contact Plate Method in the Presence of Serum.

Anti-bacterial activity of cloth in the presence of sheep serum was evaluated with the Contact Plate Method. In one series of tests, 10% serum was added to tellurite glycine agar. In another series, the test cloths were dipped into serum prior to placing them onto the surface of inoculated tellurite, glycine agar. In another series, the test cloths were dipped into serum prior to placing them onto the surface of inoculated tellurite glycine agar. With both procedures, the results were comparable to those obtained in the absence of serum.

4. Use of Contact Plate Method against Bacteria Other Than Staphylococci.

An attempt was made to apply the principle of the Contact Plate Method to enteric organisms. Selective media, including eosin methylene blue agar, S.S. agar, and brilliant green agar were inoculated with cultures of *Proteus, Salmonella, Pseudomonas, Escherichia,* and *Aerobacter* species. Test swatches were pressed against the agar as previously described. In each instance, the fabric, during incubation, absorbed the dye from the medium and made it impossible to determine the presence or absence of growth under the test cloth, although where a zone of inhibition was present it could be distinguished. The AATCC test procedure was applicable to testing activity against these organisms, but with the same limitations as were encountered in the evaluation of anti-staphylococcal activity.

DISCUSSION

The results indicate that the method of Schwartz *et al*[5]—is satisfactory for evaluating the anti-staphylococcal activity of fabric when sufficient concentration of agar-diffusible material is present to give a zone of inhibition. If, however, growth inhibition is limited to the interface between the fabric and the medium, the results are equivocal. The Contact Plate Method allows determination of effects both at the agar-

* The lauryl pyridinium salt of 5-chloro-2 mercaptobenzthiazole, manufactured by R. T. Vanderbilt Co., Inc.

fabric interface and in the agar zone surrounding the cloth. The reduced tellurite produced during staphylococcal growth response does not diffuse in the agar; the contrast between the black areas of growth and the clear areas of inhibition is marked.

Chapin[1] has recently critized the AATCC test stating that close agreement on results between different laboratories has been obtained on samples with high concentrations of germicides but not on samples with small amounts of active material. Here the implication is that the size of the zone of inhibition is directly related to the concentration of anti-bacterial agents in the fabric. While this is essentially correct, the size of this zone is also related to the diffusibility of the material in agar. As Chapin has indicated, the main area of interest is the ability of fabric to inhibit growth in the interstices of the fabric itself. Stuart[6] has discussed the difficulties inherent in interpreting the significance of agar diffusion results. Certainly, a satisfactory assay procedure must determine the status of growth both under and around the test fabric.

The importance of testing fabric was recently emphasized to us when diapers from hospitals using three different rinse additives, were tested in our laboratory. None of the three showed any anti-staphylococcal activity. Yet, when the same products were used according to the manufacturers' directions, each produced fabric with excellent anti-staphylococcal activity. Thus the three hospitals were failing to obtain the protection which these products could afford. Cooperation between the laundry, the laboratory, and the supplier would no doubt correct these difficulties.

It is apparent that improved methods for testing activity of fabric against organisms other than staphylococci are needed.

SUMMARY

A new method for testing the anti-staphylococcal activity of germicide-treated fabric was described. The procedure gives results that are more easily interpreted than those obtained with the Agar Plate Method adopted by the American Association of Textile Chemists and Colorists.

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PLACEMENT SERVICE FOR CYTOTECHNOLOGISTS

At the request of the Coordinating Committee for the expansion of Cytology of the American Cancer Society, the Executive Committee of the College of American Pathologists has authorized the conduct of a Placement Service for Cytotechnologists by the College office.

This service will function in essentially the same manner as the Placement Service for Pathologists.

Registered Cytotechnologists who have completed the course of training satisfactorily in an approved school of Cytotechnology, and who are interested in new positions are invited to apply to the College. After they have furnished certain basic information relative to training, etc., a list of openings for Cytotechnologists will be mailed to them. Supplemental lists will be sent at monthly intervals until they have obtained a position, or for six months, whichever is earlier.

Pathologists who are in need of the services of a cytotechnologist are invited to register the position with the college. The position will then be placed on the list furnished to the Cytotechnologists. Those having positions published will be placed on the list furnished a monthly listing of Cytotechnologists seeking a position—until their position is filled, or for six months, whichever is earlier.

Members of the College who are interested, will, on request, be furnished a form on which they may list the information about a vacancy.

Application should be made to the College of American Pathologists, 2115 Prudential Plaza, Chicago 1, Illinois.

ERYTHROPOIETIN: THE RED BLOOD CELL REGULATING HORMONE*

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For years scientists have been searching for the delicate mechanism that controls red blood cell production. As early as 1906 Carnot and Deflandre suggested that a factor was present in the blood plasma of anemic rabbits which, when injected into normal rabbits, increased red blood cell production. Originally the name hemopoietine had been applied to this substance but Bonsdorff (1948) renamed it "erythropoietin" because it is involved in the formation of red blood cells only. Hundreds of articles have been written confirming the presence of erythropoietin in the plasma of anemic animals. In 1952, the available reports were summarized by Grant and Root. Although there was much conflicting data, there was general agreement that a humoral substance is present in the blood plasma which affords an excellent medium for studying the mechanism that controls erythropoiesis. Bonsdorff's report (1948), as well as those by Reismann (1950), Erslev (1953), Stohlman (1954) and Gordon (1954), stimulated anew the interest in the role of this humoral substance in erythropoiesis. Classifying it as a hormone is justified because it is a chemical substance secreted into the blood by one or more organs and stimulates activities in another organ (Jacobson 1960).

For several years Dr. Jacobson, of the University of Chicago, has been actively engaged in investigating the mysteries surrounding this hormone. About 3 years ago, he and his co-workers published the results of research on the mechanisms controlling production of the hormone including extremely important evidence suggesting that it originated in the kidney (Jacobson et al 1957).

Animal Studies

The work that led to this discovery was undertaken by two medical students under the direction of Dr. Jacobson (Plzak et al 1955) (Fried et al 1956). These workers bled rats to produce an anemia, and then injected the plasma from these animals into rats that had been hypophysectomized ten days earlier. Using radioiron uptake as a basis for evaluation they found that erythropoiesis was increased by a factor of 5 to 10 in rats that received injections of anemic plasma, as compared with rats that received injections of normal plasma. Hypophysectomy has the effect of reducing rather abruptly the metabolic requirement for oxygen, and this eventually stabilizes at a level considerably below the normal. By eight to ten days after hypophysectomy the red cell mass has not fallen appreciably. These workers postulated that, as a consequence, normal erythropoietin production and so erythropoiesis, is drastically reduced in these rats. They suggested that this reduction in the production of erythrocytes, coupled with the slow rate of destruction of surviving red calls, had been brought about by the discrepancy between the demand of the tissues for oxygen and the supply of oxygen available, thus setting up a new state of equilibrium. In other words,

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**Operated by the University of Chicago for the United States Atomic Energy Commission.

conditions that decrease the demand for oxygen while the supply is normal, and those that increase the supply while the demand is normal, all produce a profound decrease in erythropoiesis. Under such conditions, the injection of erythropoietin-rich plasma produces an exaggerated response in terms of red blood cell production. Conversely, if there is an increased demand for oxygen and a normal supply (as in a hypermetabolate) or a decrease in the supply while the demand remains normal (as in anemia), erythropoiesis is increased. The starved rat also serves as a good illustration of a reduced demand for oxygen associated with a normal supply. These animals have a decreased rate of red blood cell formation, and therefore they respond to injections of anemic plasma. Another animal that responds to erythropoietin-rich plasma is the transfusion-induced polycythemic mouse (Jacobson et al 1959 and 1960); here there is an increased supply of oxygen with a normal demand. Again, rats subjected to an atmosphere simulating that of an altitude of 21,500 feet where the oxygen supply is lower than the normal demand have an increased rate of erythrocyte production, and demonstrate this by a decreased response to injections of anemic plasma, as shown by radioiron uptake (Jacobson et al 1959).

These examples serve to show that as the rate of erythropoiesis increases, the response to injections of erythropoietin decreases, and vice versa. This observation led the workers to postulate that the dynamic equilibrium of red cell production is controlled by the relationship of the oxygen supply in the tissue to the demand for oxygen and to neither alone.

The stimulating effect of erythropoietin on red cell production is shown not only by iron-uptake studies, but also by the reticulocyte response (Jacobson et al 1957). An increase in reticulocyte values is an excellent indication of the degree of stimulation produced by various types of plasma. Although counting reticulocytes is a more tedious procedure than measuring the iron uptake in red cells, it is considered to be a more sensitive technique, especially where the response is slight. In mice with a transfusion-induced polycythemia, for instance, red blood cell production is reduced to an absolute zero baseline within a few days. These animals remain polycythemic, with hematocrits of approximately 70 per cent, for periods sufficiently long to test the slightest stimulating effect. As a matter of fact, by repeated transfusions this polycythemic state can be maintained for months.

Up to this point, of course, while the investigators were looking for an assay method that would be practical for studying the elusive qualities of erythropoietin, or the source thereof, they were only corroborating evidence for its existence as previously reported by many other workers. During these studies, they found that plasma erythropoietin is elevated in animals after a single dose of cobaltous chloride (Goldwasser et al 1958), or after a massive hemorrhage (Jacobson et al 1960). This information and the sensitive assay methods described above enabled Jacobson and his co-workers to investigate the site of erythropoietin formation more effectively. They collected plasma from rats and rabbits about 12 hours after the removal of various organs such as the spleen, thymus, adrenal, ⅞ of the liver, thyroid, gonads, pancreas, stomach, intestines,

and kidneys. Immediately after the operation, the animal was either acutely bled, or was given cobalt. It was found that the capacity to respond to cobalt or a massive hemorrhage was retained by all animals, except those that had been subjected to bilateral nephrectomy (Jacobson et al 1957). These findings were subsequently confirmed in bilaterally-nephrectomized mice (Jacobson et al 1959).

The exact role played by the kidney in its association with the production of erythropoietin remains a mystery. That the increase in urea nitrogen caused by nephrectomy suppresses the production of erythropoietin can be ruled out, since no such suppression occurs when the ureters are ligated, even though the urea nitrogen values are equally elevated. It is possible of course, that the kidney does not produce the factor; it may be produced elsewhere, to be activated by the kidney in response to an anemia or hypoxia. Although this response to nephrectomy has been corroborated by Naets (1958) in dogs, by Osnes in mice (1958) and by Kuratowska (1960), Erslev (1958) found no demonstrable erythropoietin elevation after bleeding uremic rabbits. Mirand and Prentice (1957) however, found a high titer in the plasma from nephrectomized rats after exposure to low oxygen tension. It is possible, of course, that there is more than one erythrocyte-producing factor in the body, and that its production is not exclusively regulated by the kidneys. It may be that some toxic action, resulting from the nephrectomy, suppresses erythropoietin formation at some site other than the kidney. It may be produced elsewhere as an inactive precursor, which is subsequently activated by the kidney, or the kidney may produce erythropoietin in an inactive state while some other tissue activates it. Erslev (1959) and Alpen and Cranmore (1959) concluded that erythropoietin acts on the "stem cells" of the marrow producing differentiation into the red cell series.

Filmanowicz and Gurney (1960) have also shown that erythropoietin induces an orderly differentiation and maturation of the erythrocyte precursors. The rate of production is regulated by the plasma titer of the erythropoietin. Thus, in anemia, erythropoiesis is indirectly stimulated by the increased liberation of erythropoietin into the plasma.

Clinical Studies

The clinical implications are enormously important. Perhaps they can be explained in the following manner. The red blood cells carry the oxygen to all parts of the body. In normal individuals, 2.5 million red blood cells are produced each second and an equal number are destroyed. In persons with an anemia, destruction exceeds production. In these cases, erythropoietin must maintain the red blood cell balance just as insulin controls diabetes. This has been demonstrated by Gurney (1960) at the University of Chicago, who is an associate of Dr. Jacobson. He has found that erythropoietic activity is present in the plasma of patients suffering from most anemias; pernicious, hemolytic, hypoplastic, Hodgkin's disease, etc., but he was unable to demonstrate such activity in the plasma of patients with an anemia resulting from uremia or advanced neoplasia. Over 200 plasma specimens from anemic subjects have been tested and an increase in erythropoietin was demonstrated in most patients with hemoglobin values under 9 grams, with the exception

of those having advanced renal disease. These findings are in agreement with those of Gallagher and his colleagues (1959) who also could demonstrate no increase in erythropoietin titer in anemias associated with chronic renal disease.

Thus, clinical studies also indicate that the kidney plays an important role in erythropoiesis. Additional research is necessary to demonstrate the manner and extent of its participation in the process.

It is possible that anemias of malnutrition, chronic infection and rheumatoid arthritis are due to an erythropoietin deficiency, unfortunately, the assay methods known today are not sufficiently sensitive to detect this.

Although chemical and physical properties of plasma erythropoietin fractions have now been described by Dr. Jacobson's co-workers (White et al 1960), any discussion relative to these fractions, is beyond the scope of this paper. It is interesting to note that the product obtained after 50,000-fold purification is not yet the pure hormone. Large-scale production has been established using active plasma obtained from sheep, but the cost connected with these operations is enormous. This, of course, makes treatment with erythropoietin prohibitive at this time. However, the purified hormone is being released for experimental studies in the animal and human being.

Summary

It is gratifying to know that animal experimentation and clinical investigations have supported one another in these studies. We can conclude then that:

1. Hypophysectomized rats, starved rats, and transfusion-induced polycythemic mice provide the most sensitive biological assay system for erythropoietin.

2. The production of erythropoietin is regulated by the tissue oxygen supply and demand relationship.

3. Erythropoietin has been demonstrated in the plasma of many anemic patients, but not in patients having advanced renal disease.

4. The kidney appears to be the principal site of erythropoietin production.

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PHOTOELECTRIC DETERMINATION OF PROTHROMBIN TIME USING SIMPLASTIN*

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and
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The increasing use of anticoagulant therapy is placing an even greater demand upon the already busy laboratory. Since the amount of anticoagulant administered usually bears a relation to the level of the prothrombin complex, the laboratory must attempt to eliminate any variable which will cause inaccuracies in its determinations. Two main causes for variability are the thromboplastin preparation and the second is operator variability.

This communication illustrates how an attempt is made in this laboratory to control both.

The first variation was eliminated by the use of a commercially prepared thromboplastin, containing calcium, in which particle number, and particle size[1] as well as pH and salt concentration are controlled to give high reproducibility. The second variable was lessened by the use of a photoelectric colorimeter. Particularly in the "therapeutic level" of anticoagulant therapy it is frequently difficult to see the end point. This is especially true when one individual is responsible for a variety of work rather than just specializing in one.

The photoelectric technique is based upon the "increasing density of the coagulating plasma decreases the transmission of light."[2]

In practice the indicator light on the instrument moves to the left at the moment of the clotting of the plasma. When the solid clot was formed, the movement stopped. The initial movement of the indicator was taken as the end point. In our hands, the photoelectric method gives slightly shorter times as well as lower standard deviation as compared to the one-stage Quick method.

Materials:

1. Spectrophotometer—Coleman Jr. Model 6A
2. Semi Micro Adapter
3. Cuvettes 6 mm dia. 7.5 cm in length
4. 0.1 cc micro pipettes—narrow Stancien
5. 0.1 M Sodium Citrate 2.94 gms reagent "Sodium Citrate $2H_2O$ in distilled H_2O. (End point is more distinct, With plasma from citrated blood than with oxalated blood)
6. Simplastin, Warner-Chilcott
7. Diagnostic Plasma, Warner-Chilcott

Procedure:

1. Turn Coleman on to warm up
2. 4.5 cc of blood are added to 0.5 cc of 0.1 M Sodium Citrate[3]
3. Prepare Simplastin as per instructions and put in 37° C water bath for 3 minutes.

* Received for publication February 1961.

4. Centrifuge blood tubes at a medium speed for approximately 3 minutes
5. Pipette 0.1 cc of supernatant plasma into the small cuvette and put in 37° C water bath for 3 minutes
6. Put the cuvette into the semi-micro adapter of spectrophotometer and blow in 0.2 cc of prepared Simplastin. Exactly when the Simplastin is added, the stop watch is started
7. The cuvette was removed and quickly shaken three times and placed back into the adapter. *(Note:*—The light beam moved back into the scale and came to a standstill)
8. *As soon as the Galvanometer begins to move to the left, the stop watch is stopped. The amount of seconds on the stop watch is the Prothrombin Time.*

Note: The Setting of the Spectrophotometer

1. Set wave length at 525 mu
2. Turn Colorimeter switch on
3. With both the fine and coarse galvanometer controls turn completely to the left, the light beam is set at 2.0 (extreme left, top red, optical density scale)
4. Then turn fine control completely to the right and set coarse control until the light beam is barely visible on the right. Now the colorimeter is ready for operation.
5. Before turning the machine off, turn both the fine and coarse controls to the left.

Results:

Compiled from four weeks study of prothrombin time. Twelve samples of each plasma were used ($N = 12$). The following are ranges of standard deviation for the normal, medium and prolonged prothrombin time.

1. Normal Range—12.4 secs to 14.0 secs: S.D.—0.16 secs—0.21 secs
2. Medium Range—21.2 secs to 23.3 secs: S.D.—0.19 secs—0.21 secs
3. Prolonged Range—30.4 secs to 33.4 secs: S.D.—0.24 secs—0.31 secs

Discussion:

The one stage prothrombin time has been accepted as adequate in the control of anti-coagulant therapy. One of the major causes of its questionable accuracy is the inherent difficulty that may arise with the use of a preparation of variable activity.[4] Since the activity is dependent upon many factors, it is generally beyond the control of the routine hospital laboratory to exercise all of the controls necessary for the preparation of thromboplastin whose activity could be assured beyond a reasonable doubt from one time of preparation to another.

Many thromboplastin preparations have been examined and Simplastin has been found to be the only preparation that would consistently give an extremely low standard deviation.

The second source of variability in prothrombin time determination was the demand that the operator pick some point in the coagulation of the plasma as an end point, and then attempt to reproduce this end

point on each successive determination. Some authors recommend the forma-
tion of a fibrin strand as an end point, while others watch for gel forma-
tion and still others can see a pre-granulation before the fibrin strand
formation. It becomes increasingly difficult for one operator to repro-
duce the exact end point of another. The use of a photoelectric cell
which may not perhaps pick the exact end point, will however reproduce
the same end point each time. The instrument can reproduce itself. The
degree of reproducibility this laboratory has obtained, in its pro-
thrombin time determination has exceeded that which we were capable
of getting with either the "tilt tube" on the "wire loop" method for pro-
thrombin time determinations.

Summary:

A method has been presented for the determination of prothrombin
times that will assure the laboratory of a high degree of reproducibility.
The method is based upon a standardized thromboplastin preparation
containing calcium to eliminate reagent variability and a photoelectric
colorimeter to lessen operator variability.

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